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11073

A Rapid Micro Method for Determining Diodrast and Inorganic Iodide Iodine in Blood and Urine.*

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The present paper describes a rapid micro method for the determination of diodrast or inorganic iodide iodine in whole blood, blood cells, plasma or urine. Hundreds of determinations of diodrast and inorganic iodide added to water, to urine and to filtrates have shown substantially a 100% recovery. With the method in its present form not all of the iodine is recovered from some other iodine compounds investigated, skiodan, iodeikon and isoiodeikon. The method has not been applied to normal blood or urine. The diodrast and skiodan

* This work was aided by a grant from the Commonwealth Fund.

used in this work were kindly furnished by the Winthrop Chemical Company; the iodeiokon and isoiodeikon by the Mallinckrodt Chemical Company.

Principles of Method. The iodine-containing sample is digested in an acid solution of potassium permanganate, the iodine thereby being oxidized to iodate and the organic matter destroyed. The excess permanganate is reduced by addition of nitrite and the excess nitrite then destroyed by urea. The iodate is then titrated against a standard 0.0004715 N thiosulphate solution in the presence of an excess of potassium iodide.

Procedures. With urine the determination is carried out on a diluted sample. With plasma it may be done either on 0.1 cc (or less, depending on iodine content) of plasma or on a sample of plasma filtrate. Because of their higher organic matter content it has not been found practicable to digest unprecipitated whole blood or cells; these determinations must be done on filtrates, where most of the organic matter has been removed. While determinations can be carried out on unprecipitated plasma, we usually use plasma filtrates, particularly at low iodine levels, since with these an amount equivalent to a larger amount of plasma can be used. With urine no precipitation is ever required.

Procedure for determination on filtrates of blood, plasma or cells.

Reagents: 7% and 15% trichloracetic acid.
0.4M potassium permanganate.
4N sulphuric acid.
1.0M sodium nitrite.
5.0M urea.
Crystalline potassium iodide.
1% starch.
0.0004715N sodium thiosulphate.

Precipitation. Take one volume of blood, plasma or cells, 6 volumes of water and 3 volumes of trichloracetic acid (7% for blood or plasma, 15% for cells) in a small Erlenmeyer flask, shake, let stand 5 minutes and filter. On working with blood or cells, the cells are laked by the water before the trichloracetic acid is added.

Digestion. (a) *Individual tubes by hand.* Take 3 cc of filtrate in an 18 x 150 mm test tube, add 6 drops (0.3 cc) of 0.4M permanganate and 2 drops (0.1 cc) of 4N sulphuric acid. With very low iodine levels (less than 1 mg I per 100 cc) 5 cc of filtrate may be used, in which case 0.5 cc of 0.4M permanganate and 3 drops of 4N sulphuric acid are added. With high iodine levels, if less than 3 cc of filtrate is used, the volume should be brought up to about 3 cc by adding distilled water before the digestion; here 2 or 3 drops of 4N

sulphuric acid and 0.3 cc of 0.4M permanganate are used. With constant shaking, heat for 3 to 3.5 minutes over a micro Bunsen burner with flame 3.5 to 4.5 cm high and protected from drafts. Shaking should be a fine wrist motion rather than a slow undulation; keep digest constantly agitated to avoid local overheating. Boiling should begin in about 40 seconds and should then be almost continuous, with tube being continually brought into and out of flame and not in the flame more than half the time; the digest should never be permitted to boil more than one-third the distance up the tube. Avoid heating on side of tube above fluid line. After digestion add 4 or 5 drops (0.25 cc) 1.0M sodium nitrite (to 1 drop excess) drop by drop to hot digest; drop nitrite directly into digest, not running down wall. Digest will clear immediately on shaking. Heat with shaking for 1 minute, shaking so as to wash down above highest level reached by digest during digestion. Next add 4 drops 5.0M urea. Shake and heat for 2 minutes; this includes time required for tipping and rolling tube to wash down above highest level reached by nitrite. During the nitrite and urea treatments the solution should be boiled gently part of the time.

(b) *Digestion of number of tubes simultaneously in water bath.* Put tubes in a rack into a boiling water bath for 10 minutes; it is not necessary to shake during this interval. Level of water in bath must be above level of digest in tubes. Tubes need not be covered; with appropriate size of flame no difficulty should be encountered by water from bath splashing into tubes. If rack is suspended with its bottom slightly above bottom of bath, danger from bumping and splashing is abolished, although this precaution is usually not necessary. Preparation of tubes for digestion is same as with individual digestions. With either hand or water bath digestion the permanganate should not be added until shortly before digestion begins. After 10 minutes lift rack from bath and add nitrite, shaking each tube on addition of nitrite. Return rack to bath for 2 minutes. During this 2 minutes the analyst continuously makes the rounds of the tubes, lifting out a tube in each hand, giving them a shake, returning them to the bath and passing on to the next pair. With 10 tubes one should get around 3 or more times in 2 minutes. After this 2 minutes again remove rack from bath and add urea, with appropriate shaking and washing down, to each tube. Again return rack to bath for 2 minutes, with seriatim shaking as during the nitrite treatment. Finally remove rack from bath, shake tubes once more, and cool.

Titration. Put tubes in a rack into a cold water bath (5-10°C) for a few minutes. After cooling, remove from water and titrate

in an artificially lighted room, using a photoflood bulb or other source of white light. For titration, add a few crystals of potassium iodide and titrate with 0.0004715N thiosulphate, 1 drop of starch solution being added toward end of titration. Each microgram of iodine titrates 0.1 cc. The mean titration error in a triplicate series is not more than 0.003 cc, the maximum not more than 0.005. A blank on normal filtrate should show zero titration. We use a burette graduated to hundredths of a cc and read to thousandths with a hand lens. The burette tip is washed with 2 or 3 drops of water from a medicine dropper after each delivery near the end of a titration.

Reactions. The permanganate oxidizes the organic matter and carries the iodine to iodate. The nitrite destroys the excess permanganate and manganese dioxide, forming the manganous salt of the acid present. At proper acidity and with reasonably judicious treatment, *i. e.*, avoidance of excessively vigorous or lengthy heating, the nitrite does not at all reduce the iodate.

$$2KMnO_4+5HNO_2+3H_2SO_4=K_2SO_4+2MnSO_4+5HNO_3+3H_2O$$

The excess nitrite remaining after permanganate is destroyed would itself liberate iodine from potassium iodide; it must, therefore, be destroyed before the titration. While nitrite reduces permanganate it oxidizes urea, with destruction of both nitrite and urea. The excess urea remaining after destruction of the nitrite does not interfere with the titration, nor does it reduce iodate.

$$CO(NH_2)_2+2HNO_2=CO_2+2N_2+3H_2O$$

Procedure for determination on urine. With urine, dilute so that 3 cc contains from 5 to 15 micrograms iodine. Take 3 cc of dilution in test tube, add 0.3 cc of 0.4M permanganate and 0.15 cc of 4N sulphuric acid and proceed as with filtrate above, except that with water bath digestion only 5 minutes of digestion are required. A normal adult with a surface area of 1.73 sq. M. and with a plasma level of 2 mg diodrast iodine per 100 cc will have a urinary output of 8 to 12 mg iodine per minute.

We have carried out several hundred determinations of known amounts of diodrast and of potassium iodide added to water, to urine and to filtrates. In these filtrate analyses the diodrast or iodide was added to the filtrates; they are distinct from the determinations presented in Tables II and III, where diodrast was added to blood, plasma or cells before precipitation. There is a complete recovery from water and urine and from plasma, blood and cell filtrates of human, dog and horse bloods. A few representative findings are shown in Table I. These results have been obtained with amounts of iodine in the sample analyzed ranging from 2 to 15 micrograms; there may be a slight loss of iodine when more than 15 micrograms

TABLE I.
Recovery of Diodrast and Inorganic Iodide Iodine from Water, Urine, and from Filtrates.

Material	Iodine in sample analyzed, μg	Iodine recovered, μg	% recovery
Aqueous solution of diodrast	6.00	6.00	100.0
	6.00	5.96	99.4
	8.00	8.04	100.5
	12.00	11.90	99.2
	12.00	12.02	100.2
Diodrast in urine	9.27	9.32	100.5
	9.20	9.15	99.5
" " human plasma filtrate	6.00	6.04	100.7
	6.00	6.00	100.0
	12.00	11.93	99.4
" " whole blood filtrate			
human	6.00	6.08	101.2
horse	8.00	7.91	98.9
Potassium iodide in horse whole blood filtrate	6.00	6.04	100.7

is taken. We can, therefore, say that the above procedure will determine quantitatively the iodine present in the sample analyzed. With urine this means all the iodine present but with blood, plasma or cell filtrates the question arises whether all the diodrast comes through in the filtrate. This point has been investigated at some length on horse, dog and human bloods.

The percentage of diodrast coming through in the filtrate has been determined at various iodine levels for whole blood, for plasma and for cells. The outline of a typical experiment follows: With a given sample of blood, 3 trichloracetic precipitations are made on whole blood, 3 on plasma and 3 on cells, where a known amount of diodrast has been added to the blood, the plasma and the cells, respectively, before precipitation. From 3 to 6 iodine determinations are made on each of the 9 filtrates so obtained. Whole blood and cells are delivered from pipettes to contain and are laked before addition of diodrast. The above procedure is carried out with each of 3 known iodine concentrations. Table II shows the results of such an experiment with a sample of horse blood. Each value in the table represents the average of the 3 to 6 determinations on that filtrate; the maximum deviation from the mean among determinations on a single filtrate is never more than 3%, with a probable error of less than 2% in a triplicate series.

Experiments of the type outlined in Table II have been carried

TABLE II.
Horse Blood 2.
Figures in the Table Designate the Percentage of Diodrast Coming Through in the Filtrates.

		Whole blood	Plasma	Cells
At 2.5 mg I per 100 cc	Filtrate 1	67.0	82.0	49.8
	" 2	64.5	81.2	51.0
	" 3	65.7	82.0	
At 5 mg I per 100 cc	" 1	66.9	82.2	50.6
	" 2	65.2	82.8	51.3
	" 3	66.9	84.3	
At 10 mg I per 100 cc	" 1	64.8	82.5	
	" 2	65.1	81.7	
	" 3	66.1	81.8	
Avg % of diodrast coming through in filtrate		65.8	82.3	50.7

out on 4 horse, 2 dog and 5 human bloods, at iodine levels of 2, 2.5, 4, 5, 8 and 10 mg per 100 cc. It was found that the percentage of diodrast appearing in the filtrates was independent of the iodine concentration within the above range. It was further found that not only is the percentage of diodrast coming through in the filtrate constant with multiple precipitations from a given sample of blood, plasma, or cells but that it is almost constant in all normal horse, dog and human bloods so far examined. The findings are shown in Table III, where a given figure designates the average of the findings on all the filtrates of a given sample of blood, plasma or cells.

The above findings show that with normal human bloods no great error will be introduced by the assumption that the diodrast iodine

TABLE III.
Percentages of Diodrast Coming Through in Whole Blood, Plasma and Cell Filtrates of Horse, Dog and Human Bloods.

	Whole blood	Plasma	Cells
Horse 1	64.3	84.3	
" 2	65.8	82.3	50.7
" 3	63.9	84.5	
" 4			50.8
Dog 1	65.0	85.2	49.4
" 2	69.4	84.2	54.8
Human			
D.N.	66.0	85.0	48.0
Grov.	64.4	84.0	48.3
Loe	64.8	84.0	
W.C.S.	66.9	85.5	54.4
E.W.S.	65.8	84.6	55.3
Avg of humans	65.6	84.6	51.5

content of whole blood is 100/65.6 × that indicated by the filtrate analysis; for plasma the factor is 100/84.6 and for cells 100/51.5. It is of course a simple matter to determine these factors directly for each subject under investigation if the highest accuracy is desired. Since urine is digested without precipitation, all of the iodine in the sample is determined and no factor is used. The calculation here is simple, *i. e.*, each 0.1 cc of titration represents 1 microgram of iodine in the sample analyzed.

Alternative procedure for determination of iodine in plasma. As stated above, the determination may be made on 0.1 cc or less of unprecipitated plasma. In view of the constancy of the percentage of diodrast appearing in plasma filtrates we prefer to use the filtrate procedure, particularly if the level is below 2 mg per 100 cc, since 3 cc or more of filtrate can be used, giving a higher titration. However, the alternative procedure with iodine levels of 2 mg per 100 cc or more can be used, although it is considerably less accurate.

To 1 volume plasma or serum add 19 volumes water. Take 2 cc of this dilution in a 22 x 175 mm test tube, add 1 cc 0.4M $KMnO_4$ and 0.5 cc 4N H_2SO_4. With high diodrast levels correspondingly greater dilutions can be made. Digest 3 to 3.5 minutes by hand. Add 1M nitrite to hot digest drop by drop, shaking with each drop addition. Add 2 drops excess; the total will be 8 to 10 drops. More time will be required in washing down the tube walls than is the case with filtrate, since in the present case there is a greater amount of MnO_2 to be destroyed. The nitrite treatment, including addition of nitrite, shaking, washing down and heating, will take 2 to 3 minutes. Next add 7 drops (0.5 cc) of 5M urea, heat, shake and wash down for another 2 minutes. Titrate as with filtrate. Along with each set of analyses carry out a determination on a known amount of diodrast added to a blank plasma and use the factor so obtained in calculating the unknowns. The departure of this factor from unity will in general be considerably less than with the alkali fusion method of iodine determination. It departs somewhat from unity because, (a) with a less vigorous nitrite treatment a trace of MnO_2 remains undestroyed, raising the titration, (b) with a more vigorous nitrite treatment, sufficient to give a very low blank, small amounts of iodine are likely to be lost (iodate reduced to iodide), lowering the titration. The factor for a given analyst varies within narrow limits; different analysts may show different factors.

With the unprecipitated plasma digestion the error should not be greater than 10%; with continued practice the error can be reduced below this figure but the accuracy attainable with this method will never be as great as with filtrates.

11074 P

Interpretation of Diodrast Clearances in the Dog.*

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If some otherwise suitable substance could be found which was completely removed from the blood on one passage through the kidneys, its blood clearance would be a measure of renal blood flow. If the substance were either (a) absent from the blood cells or (b) fixed in the cells during a passage through the kidney, its plasma clearance would be a measure of renal plasma flow (RPF). Diodrast (D) has been proposed by Smith and collaborators[1, 2, 3] as meeting or very nearly meeting the conditions that it is completely removed on passing active renal tissue and that it is absent from the cells. We find on trained, unanesthetized renal explant dogs, with D[4] and inulin[5] analyses of urine and of renal vein and arterial (or leg vein) whole blood, plasma and cells, that D plasma clearance departs considerably from RPF independently measured and we have established the factors which bring about this discrepancy.

1. *Diodrast in cells.* The statement of Smith[1] that D is absent from dog cells is based upon essentially complete recoveries from plasma of D added to drawn blood. We confirm this finding but find that after intravenous administration the ratio

$$\frac{\text{D per 100 cc arterial cell water}}{\text{D per 100 cc arterial plasma water}}$$

averages 0.62, with extremes 0.48 to 0.73. The average is of 18 observations with plasma levels from 1.51 to 11.50 mg I per 100 cc; the value of the ratio is independent of plasma iodine (I) level, I being a measure of D.

2. *Diodrast contribution to urine by cells.* Even though we have shown that the cells contain a large proportion of the D, one could still use D plasma clearance as a measure of RPF (if plasma D extraction were complete) provided all the cell D stayed in the cells on

* Aided by a grant from the Commonwealth Fund.

1 Smith, H. W., Goldring, W., and Chasis, H., *J. Clin. Invest.*, 1938, **27**, 263.

2 Chasis, H., Ranges, H. A., Goldring, W., and Smith, H. W., *J. Clin. Invest.*, 1938, **27**, 683.

3 Smith, H. W., *Physiology of the Kidney*, Univ. of Kansas Press, Lawrence, 1939.

4 White, H. L., and Rolf, D., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 1.

5 Corcoran, A. C., and Page, I. H., *J. Biol. Chem.*, 1939, **127**, 601.

passing the kidney. If, however, cell D moves into plasma on D depletion of the latter, then urinary D per minute will be greater than plasma contribution of D per minute and to this extent, if there were no other complicating factors, D plasma clearance would exceed RPF. We have shown that such a contribution of cell D does take place; renal vein cell D is always much lower than arterial cell D. The average ratio

$$\frac{\text{mg I per 100 cc renal vein cells}}{\text{mg I per 100 cc arterial cells}}$$

is 0.74 (average of 9 observations). However, renal vein cell D does not fall proportionally to renal vein plasma D; whereas arterial plasma D is always higher than arterial cell D, renal vein plasma D is always lower than renal vein cell D. The above observations show that D does not behave (a) as does inulin, since D is present in the cells, (b) as does creatinine, since some D moves from cells into plasma during a renal passage, with a partial approach toward diffusion equilibrium, nor (c) as does urea, since D does not come into complete diffusion equilibrium between cells and plasma during a renal passage.

3. *Completeness of extraction of diodrast from renal vein blood or plasma.* We find that renal extraction $\frac{A - RV}{A}$ of D is never complete from either plasma or cells, with arterial plasma levels from 1.52 to 7.09 mg I per 100 cc. Plasma D extraction averaged 0.73 (0.61-0.85), cell extraction 0.24 (0.10-0.39) and whole blood extraction 0.58 (0.46-0.66). Within this range of plasma levels there is only rough correlation between plasma I level and completeness of extraction; the series is being extended.

4. *Relation between diodrast plasma clearance and renal plasma flow.* Two factors, the incompleteness of D extraction and the cell contribution of D, work in opposing directions to disturb the relation between D plasma clearance and RPF; the action of the first is to make D plasma clearance lower than RPF, while the second works to make D plasma clearance higher than RPF. If the first factor alone were operative, D plasma clearance/D plasma extraction would be a measure of RPF. Since the second factor also is always operative, this expression will always give values higher than RPF. If the cell contribution of D raises D plasma clearance less than the factor of incompleteness of D extraction lowers it, D plasma clearance will be less than RPF. It is conceivable that the two factors could be just balanced, so that D plasma clearance would equal RPF; this can be determined only by simultaneous D clearance determinations and independent flow determinations.

5. *Comparison of diodrast plasma clearances with independently determined renal plasma flows.* Let V_p equal plasma fraction of hematocrit reading. Renal plasma flows were obtained by 3 methods simultaneously,

$$(a)\ \frac{\text{Inulin plasma clearance}}{\text{inulin plasma extraction}},$$

$$(b)\ \frac{\text{Inulin whole blood clearance} \times V_p}{\text{inulin whole blood extraction}},$$

$$(c)\ \frac{\text{D whole blood clearance} \times V_p}{\text{D whole blood extraction}}.$$

The average values in cc/min/M², are (a) 360, (b) 370, (c) 355, while the corresponding D plasma clearance average is 310, and the average

$$\frac{\text{D plasma clearance}}{\text{D plasma extraction}}$$

is 406. The last value is higher than RPF because of the cell contribution of D; 406 is 113% of 362, the average true RPF, which means that for every 100 mg D contributed to the urine by the plasma, 13 mg are contributed by the cells. This is even more direct evidence of cell contribution of D than is the finding that renal vein cell D is lower than arterial cell D, since the latter finding might be explained by diffusion after drawing the sample. The D plasma clearance is lower than RPF because the factor of incompleteness of D extraction overbalances that of cell contribution of D. On the average, with the dog one can get RPF by multiplying D plasma clearance by 1.17, but this multiplier varies between 1.03 and 1.43. These values were obtained over a range of plasma I levels from 1.52 to 7.09 mg per 100 cc, with but little correlation between value of multiplier and of plasma I level. Average whole blood flow is 683 cc/min/M², or 23.2% of cardiac output, taking Marshall's[6] value of 2940 cc/min/M² for the latter. Inulin plasma clearance averages 101 cc/min/M². Both diodrast and inulin plasma clearances in the hypophysectomized dog are about 50% of normal.

[6] Marshall, E. K., Jr., *Am. J. Physiol.*, 1926, **77**, 459.

11075 P

Interpretation of Diodrast Clearances in Man.*

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A series of diodrast (D) plasma clearances has been carried out on 11 normal human subjects, with plasma iodine (I) levels varying from 0.3 to 55 mg per 100 cc; simultaneous inulin clearances were determined in some cases. Data on distribution of D betwen cells and plasma are also given.

1. *Diodrast in cells.* The statement of Smith[1] that D is absent from the cells of drawn human blood is confirmed. However, at equilibrium after intravenous administration of D the ratio

$$\frac{\text{D per 100 cc arterial cell water}}{\text{D per 100 cc arterial plasma water}}$$

averages 0.32. It must be emphasized that here, as with the dog (0.62), the cell/plasma D distribution ratio was obtained after *in vivo* equilibration had been attained. The cell content of D in man is thus about half that in the dog for a given plasma level, after cells and plasma have come into equilibrium *in vivo*.

2. *Diodrast contribution to urine by cells.* Magnitude of contribution of D to urine by cells during a renal passage will be determined by, (a) cell content of D and, (b) rapidity with which D passes from cells into plasma on D depletion of the latter. On the first of these points alone, cell contribution of D in man would be about half of that in the dog. Information on the second point has been obtained by observing the cell/plasma D ratio on a falling plasma D level following an equilibration period of a constant plasma D level, *i. e.*, by following the ratio after cessation of sustaining infusion. Such observations on the dog show a constant ratio, *i. e.*, D diffuses from cells into plasma rapidly enough, as plasma D falls, to maintain a constant distribution. With man, on the contrary, the ratio rises rapidly as plasma level falls following cessation of sustaining infusion; it may go from the equilibrium value of 0.32 to as high as 1.20. This means that D cannot pass rapidly enough from human cells into depleted plasma to keep pace with this rate of fall of plasma

* This work was aided by grants from the Commonwealth Fund (H.L.W.) and from the Smith, Kline and French Laboratories (T.F.).

1 Smith, H. W., Goldring, W., and Chasis, H., *J. Clin. Invest.*, 1938, **27**, 263.

D. The conclusion is therefore justified that the cell contribution of D in man is less than half that in the dog, *i. e.*, on the average less than 6%.

3. *Completeness of extraction of diodrast from renal vein plasma.* In the absence of direct observations on human renal vein plasma our only information here is indirect. Our data so far available suggest that D plasma clearance begins to be self-depressed at lower plasma D levels in the dog than in man, which suggests that human kidneys extract D more efficiently than do dog kidneys.

4. *Relation between diodrast plasma clearance and renal plasma flow RPF.* Since the cell contribution of D is shown to be less in man than in the dog and since the above paragraph suggests that renal extraction of D may be more nearly complete in man than in the dog, it may well be that D plasma clearance in man is a reasonably good measure of RPF. A final answer must await more adequate information on the extraction in man. Since cell contribution of D in man is shown to be small, D plasma clearance (at D plasma levels below that at which clearance begins to be self-depressed) will be less than RPF unless D is almost completely extracted. If extraction is complete in man, D plasma clearance cannot exceed RPF by more than 6%.

Values of D plasma clearance over a wide range of plasma iodine (I) levels but in the absence of inulin are shown in Fig. 1; there is no self-depression of clearance up to plasma I levels of 15 mg %. The average D plasma clearance, at D plasma levels below that at which clearance begins to be depressed, in cc/min/1.73 M^2 is 497 (58

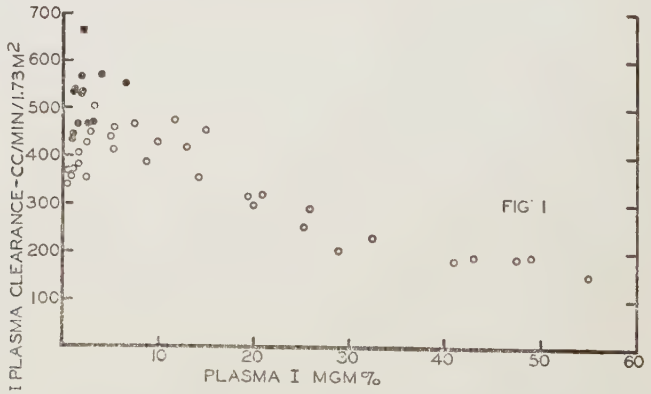

FIG. 1.

Effect of varying plasma I level on plasma I (or D) clearance. Each solid circle designates the mean of 3 or more consecutive clearance periods at a constant plasma level. Open circles designate individual clearance periods obtained on falling plasma D levels after a single injection. No inulin.

clearance periods on 11 subjects), which is considerably lower than Smith's latest figure of 737 and close to Chesley and Chesley's[2] figure of 518. The point might be raised that Smith's higher figure is due to the inulin present in his experiments. However, our average of D clearances obtained simultaneously with inulin clearances is 438 cc/min/1.73 M^2, which shows that the higher value of Smith cannot be ascribed to any action of inulin. Self-depression of D clearance does not begin until plasma I is raised to about 15 mg %, whereas Smith finds D clearance only 65% of normal at this plasma level.

Rate of tubular excretion of D is plotted against D plasma level in Fig. 2. Maximum rate of tubular excretion (Tm) averages

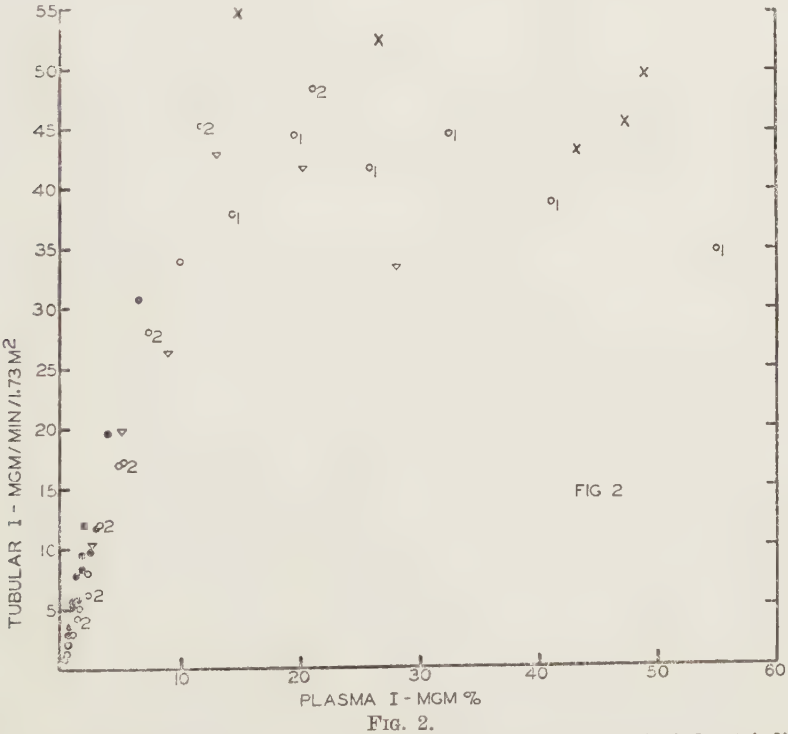

FIG. 2.

Effect of varying plasma level on rate of tubular excretion of diodrast iodine. Rate of tubular excretion is almost directly proportional to plasma level up to somewhere between 10 and 15 mg I per 100 cc plasma, where it breaks rather sharply, Tm being reached at between 15 and 20 mg I per 100 cc plasma. Solid circles designate means of 3 or more consecutive periods at a constant plasma level. ◯ individual periods on a falling plasma level after a single injection with subject TF, ◯1 same for another experiment on same subject, ◯2 same for another experiment on same subject; △ same on subject JE; × same on subject P.

2 Chesley, L. C., and Chesley, E. R., *Am. J. Physiol.*, 1939, **127**, 731.

44 mg I/min/1.73 M^2 on 3 normal subjects; it is reached at a plasma level between 15 and 20 mg I per 100 cc and is unaffected by presence of inulin. This value is somewhat lower than Smith's latest figure of 57. The fall in Tm seen in Fig. 2 at the highest plasma I levels was obtained in experiments where a single large dose of D (35 to 60 cc) was given within 10 minutes and urine collections begun 5 minutes later. This transitory fall in Tm is presumably due to a renal vasoconstriction or to transitory tubular disturbance and is not seen when plasma D is maintained at a high constant level for some time before beginning urine collections, *i. e.*, in the latter case Tm at 50 mg % plasma I is not lower than at 20 mg %.

11076

Serum Albumin Regeneration Following Intravenous Amino-Acids (Hydrolyzed Casein) in Hypoproteinemia Produced by Severe Hemorrhage.*

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Acute hypoproteinemia was produced in fasting dogs by a single severe hemorrhage; the details have been described elsewhere.[1] One hour later injections were started, intravenously, which lasted 3 hours.[2] No symptoms were produced by this treatment. The solution injected consisted of 10% each of a mixture of amino-acids and dextrose. The dose varied with the weight of the dog, *i. e.*, 3.5 g per kilo of each (amino-acids and dextrose). The amino-acid mixture was obtained from casein by enzymic digestion.† As shown by preliminary observations it contained all of the essential amino-acids in that it required no additions in order to maintain nitrogen balance in dogs. It was not as completely digested as the acid hydrolysate of casein which was used in our previous experiments.[2] Thus, only 70% of its nitrogen could be accounted for an amino-acid nitrogen; the rest probably occurred as dipeptides.

In control experiments[1] it was observed that no change in the

* Aided by a grant from the Louis B. Beaumont Fund.

1 Elman, R., *Am. J. Physiol.*, Jan., 1940.

† Product 92-Z kindly furnished by the Mead Johnson & Company, Evansville, Ind.

2 Elman, R., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 867.

serum albumin or globulin occurred from the first to the sixth hour after a standard hemorrhage and replacement with Ringer's solution. In the present experiments, however, a definite increase from 2.60 to 2.95 g % in the albumin fraction was observed. That this was not due to a concentration of the plasma was indicated by two findings: first, that the hematocrit decreased indicating a dilution rather than a concentration; moreover, the magnitude of this dilution was the same as previously reported in the controls.[1] Secondly, the globulin fraction remained relatively unchanged indicating that mechanical alterations in the plasma volume were probably not responsible for the increase in the albumin.

From these considerations it is inferred that the injected amino-acid mixture was utilized by the body in the rapid regeneration of serum albumin. The magnitude of the change was not great; it is suggested that this is probably due to at least two factors. First, it is probable that under the stress of an acute hemorrhage amino-acids are drawn upon from all tissues and this deficit must be met. There is evidence, moreover, which will be described in a subsequent communication, that large amounts of nitrogen are actually lost in the urine during the course of a severe hemorrhage. Second, the amino-acid composition of casein is considerably different from serum albumin in that a number of essential amino-acids are present in quite insufficient concentration, thus acting as limiting factors as far as the utilization gram for gram is concerned and requiring a much larger dose of casein in order to make up for the deficiency.

The methods used in the present experiments are described in another communication.[1] The findings in 6 animals are summarized in Table I. The change in serum albumin herein reported (.35 g %) compares well with the change in serum protein (.38 g %) previously reported with similar experiments in which a fortified acid hydrolysate of casein was used.[2]

TABLE I.

Changes in hematocrit in percent of whole blood (H), serum globulin in g % (Glob.) and serum albumin in g % (Alb.), one and six hours after hemorrhage and replacement.

	One-hr determinations			6-hr determinations		
Dog	H.	Glob.	Alb.	H.	Glob.	Alb.
F16	24.8	1.17	2.23	24.2	1.09	2.32
G5	29.2	1.84	2.12	26.8	1.48	2.71
G6	36.0	1.10	2.86	32.2	.87	3.21
27	53.9	2.56	3.00	50.3	2.65	3.24
28	44.3	2.19	3.03	41.7	1.90	3.62
30	44.5	2.19	2.36	44.5	2.47	2.59
Avg	38.5	1.84	2.60	36.7	1.74	2.95

Summary. The intravenous injection of a mixture of amino-acids (enzymic hydrolysate of casein) was followed in a few hours by a significant increase in the serum albumin concentration of fasting dogs rendered hypoproteinemic by a severe hemorrhage. Since the serum globulin concentration and the relative red cell volume both decreased, it is inferred that the increase in serum albumin concentration was due to a regeneration.

11077 P

Further Studies of Antigenic Properties of Bacteriophage.

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Although bacteriophage is antigenic, it has not been found possible to induce active anaphylaxis in guinea pigs with bacteriophage,[1] even when employing for the test-injection active purified bacteriophage-protein containing 2.6×10^{13} lytic units, (1.79 mg of specific protein,[2] equivalent to 5 liters of crude phage). In seeking another method of demonstrating the interaction of bacteriophage with its antibody *in vivo,* we attempted to utilize the Shwartzman test.

Four rabbits were sensitized by repeated injections of crude *B. coli* bacteriophage, and their sera, 10 days after the last (ninth) injection, were found to be capable (in a dilution up to 1:320) of neutralizing an equal volume of undiluted crude phage having a titer of 10^9 lytic units per cubic centimeter. Two of these rabbits were then prepared by the intracutaneous injection of 0.25 cc of meningococcal filtrate, kindly sent us by Dr. Shwartzman, and 24 hours later one of them received intravenously 5 cc of crude phage and the other 9.5 cc of freshly prepared active purified phage containing a total of 5.7×10^{13} active lytic units (or 3.42 mg of specific protein equivalent to 11.4 liters of crude phage).

The animal that had received crude phage responded within 3 hours with a typical reaction at the site of the preparatory injection, while the one that had received the purified phage had no reaction. This difference indicated that the reaction in the animal receiving

[1] Bronfenbrenner, J., and Kalmanson, G. M., *J. Bact.*, in press.

[2] Kalmanson, G. M., and Bronfenbrenner, J., *J. Gen. Physiol.*, 1939, **23**, 203.

crude phage might have been due not to the bacteriophage itself but rather to other constituents of the crude phage.

In order to inquire further into the nature of this response, the other 2 sensitized rabbits were similarly prepared with meningococcal filtrate, and 24 hours later one of them received 5 cc of sterile broth intravenously, and the other 5 cc of a Berkefeld filtrate of an 18-hour broth culture of *B. coli*. The rabbit receiving broth had no reaction, while the one receiving the culture-filtrate had a mild but definite reaction. Furthermore, 2 out of 3 normal rabbits gave positive reactions at the prepared sites when injected intravenously with crude bacteriophage, thus indicating the presence of a directly reacting factor in the crude lysate.

Similarly we failed to obtain a Shwartzman reaction in a test of reverse passive sensitization to bacteriophage: a normal rabbit was prepared by injection of meningococcal filtrate and, after an interval of 24 hours, was given an intravenous injection of 11.2 cc of purified active phage containing 7.84×10^{13} units (or 4.7 mg of specific protein equivalent to 15.7 liters of crude phage), followed in one-half hour by 5 cc of pooled antiphage-serum intravenously. There was no reaction.

Thus, attempts to demonstrate the *in vivo* interaction of bacteriophage with its antibody by means of the Shwartzman test were not successful in rabbits sensitized to bacteriophage either actively or passively, despite the fact that amounts of purified phage-protein equivalent to many liters of crude bacteriophage were used for the test-injections.

We have been able to carry out only few experiments thus far, because lots of 15 to 20 liters of phage yield sufficient purified phage for only one test, and because it is impossible to accumulate a greater quantity since purified phage loses its potency on standing.

11078 P

Precipitin Antisera for Malignant Tissue.

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Tissue from human gastric carcinoma was prepared according to the method of Spinka and Weichselbaum.[1] Aluminium cream[2] was used to adsorb the ground carcinomatous tissue and injected into the thigh muscles of rabbits. The resulting antisera, tested biweekly, lost precipitins for human blood-proteins within 1 to 6 months, but still reacted with autolysates of human gastric carcinoma, although not with autolysates of 2 series of 14 different normal organs.

These specific antisera were tested with sera of carcinomatous patients, of normals, and of variously diseased individuals; 67% of patients with carcinoma of the stomach gave positive reactions, but as there are several varieties of gastric carcinoma, this low percentage may be due to the fact that different types may be made up of proteins of different antigenicities. This point is under investigation. Patients with other types of carcinoma had positive sera, but not in so great a percentage. Normal sera and those from the variously diseased did not react with one exception—a patient with pulmonary abscess (carcinoma not excluded).

Antisera for carcinoma of the cervix gave 87% positive reactions with 8 sera of patients with carcinoma of the cervix. The sera of normal individuals did not react nor did sera of the various diseases with the same exception noted above. Three antisera for carcinoma of the cervix reacted with autolysates of 2 different carcinomas of the stomach and of a carcinoma of the cervix. Only one of these antisera gave a few positive reactions with autolysates of rectal carcinoma.

A precipitin antiserum for human rectal carcinoma reacted with autolysates of carcinoma of the rectum and breast, but not with autolysates of rat-carcinoma No. 256 or rat-sarcoma No. 10. Antisera for rat-carcinoma No. 256 and rat-sarcoma No. 10 reacted with autolysates of rat-carcinoma No. 256 and rat-sarcoma No. 10, but not with autolysates of human carcinoma of the breast or rectum.

Heretofore, it was difficult to prepare specific antisera for tissue-

[1] Spinka, I., and Weichselbaum, P. K., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 447.

[2] Welker, Wm. H., and Tracy, G., *J. Biol. Chem.*, 1915, **22**, 55.

proteins because antibodies for blood proteins appear no matter how well the finely ground tissue is washed—even to a negative precipitin-reaction. Spinka and Weichselbaum[1] have attempted to prepare specific antisera by the adsorptive method *(in vivo* and *in vitro)*. They found that the titer of the antiserum to the specific protein was diminished or disappeared entirely. We have confirmed their results *(in vitro)* in the case of antisera for malignant tissue. In the method outlined above, adsorption is not necessary because the antibodies for blood-proteins disappear in a shorter time than do the antibodies for proteins of carcinomatous tissue.

Antisera for carcinoma, as prepared by Lumsden,[3] Pybus and Whitehead,[4] Phelps,[5] must necessarily contain antibodies for blood-proteins as well as for malignant-tissue proteins. The blood-protein antibodies may well be responsible for the toxicity of these antisera observed by these investigators.

Conclusions. 1. Specific antisera have been prepared for the proteins of malignant tissue. 2. There is a suggestion of a relative anatomic specificity of carcinomatous tissue, as a greater number of positive reactions occur with autolysates of the homologous tissue and with sera of cases having the same type of malignancy. 3. These precipitins for the proteins of malignant tissue show promise as diagnostic aids. A high percentage of sera from patients with malignant tumors react positively. The higher percentages of positive reactions were obtained in cases where the malignant growth had the same anatomic location as the tumor from which the antiserum had been prepared. 4. Our preliminary results indicate that possibly these precipitins are species-specific, because antisera for rat-tumors do not react with autolysates of human carcinoma and *vice-versa.*

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3 Lumsden, T., *J. Path. and Bact.*, 1931, **34**, 349.

4 Pybus, F. C., and Whitehead, H. R., *J. Path. and Bact.*, 1929, **32**, 195.

5 Phelps, H. J., *Am. J. Cancer*, 1937, **31**, 441.

11079 P

Study of the Effect of Specific Kidney Antisera on the Normal Kidney.

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Pure albumin and pseudoglobulin were prepared from dog's blood according to the method of Hektoen and Welker.[1] Potent precipitins were prepared for the above antigens according to the method of Hektoen and Welker[2] and were used to determine the presence or absence of blood-proteins in the urine of dogs in subsequent experiments.

Four normal dogs with normal urines were exsanguinated; the blood was saved to obtain blood proteins, and the liver, brain, stomach, spleen, lung and kidneys were perfused with saline, and then thoroughly ground. A portion of kidney and all of the other organ-materials were used to prepare autolysates, and the remainder of the kidney-material was washed several times with more saline, fixed on aluminium cream and injected into rabbits for preparation of organ-antiserum according to the method of Spinka and Weichselbaum,[3] whereby blood-protein antibodies are eliminated without the necessity of absorption.

The kidney-antisera cross-reacted with liver- and brain-autolysates though to a definitely lower titer than with the specific antigen.

Healthy dogs with normal blood pressure and urine were injected intravenously with normal rabbit serum, a dog-kidney antiserum which had become attenuated, and a potent kidney-antiserum in doses of 1 cc per pound of body weight. Urines were examined for albumin, red blood cells, and casts, and by means of precipitin-tests for kidney-protein and dog-blood protein.

The injection of normal rabbit serum and attenuated kidney-antiserum produce no demonstrable changes. The injection of potent kidney-antiserum produced within one hour blood-pressure changes, appearance of kidney-proteins, red blood cells, and granular casts in the urine, and the microscopic pathological changes of extreme diffuse vascular dilatation, increase of Bowman's space, branching of glomeruli, tubular degeneration, and leukocytic infiltration of the entire kidney.

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1 Hektoen, L., and Welker, W. H., *J. Infect. Dis.*, 1924, **35**, 295.

2 Hektoen, L., and Welker, W. H., *J. Infect. Dis.*, 1933, **53**, 309.

3 Spinka, I., and Weichselbaum, P. K., *J. Chem. Soc.*, 1938, **38**, 447.

TABLE I.
Results of Intravenous Injection of Various Sera into Dogs.

	Normal Serum	Attenuated kidney-antisera	Potent kidney-antisera	Potent kidney-antisera
Blood pressure	No change	No change	Marked drop	Drop
Urine				
Albumin (Heat and acetic acid)	Negative	Negative	+++	+++
Microscopic				
Red blood cells	,,	,,	5-10/HPF	Packed
Casts	,,	,,	Negative	Occ. gran. cast
Precipitin-reactions for dog-blood protein	,,	,,	+	+
Dog-kidney protein	,,	,,	+	+
Necropsy findings				
Gross				
Capsule	Strips with ease	Strips with ease	Strips with ease	Strips with ease
Hemorrhage	None	None	None	Present
Microscopic				
Increased Bowman's space	Negative	Negative	Present	Present
Glomeruli	Normal	Normal	Branched	A few were obliterated
Congestion	Some	Some	Marked	Marked
Tubules	Cloudy swell.	Cloudy swell.	Gran. degen.	Gran. degen.
Leukocytes	Negative	Negative	Few	Many

These effects of kidney-antiserum free from antibodies for blood proteins suggest that its nephrotoxic effect is dependent on an antigen-antibody reaction and is not due to the primarily toxic influence of the animal serum or to the presence of antibodies to blood proteins as has been suggested by Pearce[4] and Smadel.[5]

11080 P

Preparation and Diagnostic Value of an Antiserum for Placental Protein.

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This investigation was undertaken first, because a study of the placenta might add to our meager knowledge of tissue antigens and second, a placental antiserum might afford a simple diagnostic test for pregnancy.

There are several reports on antisera for placental proteins. Kintse[1] prepared an antiserum for syncitial material, which contained a specific antiplacental precipitin. Lake[2] isolated nucleoprotein, globulin, albumin, and gelatin from human placenta and by immunization with these produced antisera that reacted with placental proteins and with human serum.

The method of Spinka and Weichselbaum,[3] utilized for the preparation of antisera, makes it possible to obtain directly, without relying on the use of absorptive procedures either *in vivo* or *in vitro,* sera free of antibodies for blood proteins.

Fresh human placentas were frozen and the cord and amniochorion were readily removed. The placentæ were ground in a meat-grinder and then washed with large volumes of chilled saline until snowy white. After further grinding with sand, the fine suspension was washed free of water-soluble protein, adsorbed on aluminium hydroxide cream, and injected intramuscularly into rabbits. After

4 Pearce, R. M., *Univ. Penn. Med. Bull.*, 1903-04, **16**, 217.

5 Smadel, J. E., *J. Exp. Med.*, 1936, **64**, 921.

1 Kintse, *Z. Geburtsch.*, 1912, **72**, 575.

2 Lake, *J. Infect. Dis.*, 1914, **14**, 385.

3 Spinka and Weichselbaum, PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 447.

2 months the serum no longer contained precipitins for human blood-proteins, presumably due to the exhaustion of the blood-proteins from the antigen-depots. Placental autolysates were stratified on the antiserum and reactions were read after one hour at room temperature. A precipitation at the interface appearing after this time was considered a positive reaction. The most potent preparations were obtained after autolyzing the tissue for 8 hours at 37°C.

The organ-specificity of the antiserum was tested with autolysates of 16 normal human organs. Autolysates of ovary, prostate, heart, testis, adrenal, lung, pituitary, pancreas, and breast gave negative reactions. Those of brain, kidney, liver, stomach, uterus, thyroid, and spleen gave positive reactions in varying degrees, those of liver, kidney, and uterus giving the strongest. These results correspond with the findings of other investigations on organ-antisera.

With an antiserum that reacted strongly with placental autolysate, we attempted to demonstrate the presence or absence of placental proteins in 160 samples of blood-sera and urines of pregnant women. Of the known pregnancies tested in the second and third lunar months (4 and 9 cases respectively), a positive reaction occurred with sera in 75% and 78%; with urines in 75% and 33%. In the later months of pregnancy comparable percentages were obtained. The sera of normal non-pregnant women and of males gave negative reactions. Urines that gave positive reactions became negative after standing for a short time and therefore the urines were tested directly after being voided.

Sera of certain gynecological patients, such as fibroid uterus, salpingitis, and cervicitis, gave positive reactions. However, with antiserum from which precipitins for liver and kidney had been removed by *in vitro* absorptive methods, these reactions disappeared. Although strong reactions were obtained with autolysates of placental tissue the reactivity of the absorbed serum had been so diminished that it was no longer capable of detecting the minute amount of placental protein present in the urines and sera of pregnant women. Investigations now under way towards the modification of our technic give promise of production of specific high-titered sera.

Conclusions. A precipitin for placental proteins reacted strongly with autolysates of placental tissue, kidney, liver, and uterus. It reacted with a high percentage of the sera of pregnant women but not with normal sera. Some women with diseased genito-urinary tracts yielded positive sera.

11081 P

Influence of Catharsis and Diarrhea on Gastrointestinal Absorption of Ascorbic Acid in Infants.

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Since the development of a method for the determination of ascorbic acid in feces by Chinn and Farmer,[1] it has been possible to study the normal and abnormal absorption of ascorbic acid from the intestinal tract. Abt and Farmer[2] have emphasized the possibility that under certain pathologic conditions absorption of vitamin C from the intestinal tract must be definitely abnormal. This hypothesis has been experimentally confirmed in the adult.[1] Meyer and Robinson,[3] in studying infants with diarrhea, noted that the blood plasma values for ascorbic acid remained low, and the urinary excretion was diminished, even though these patients were given large daily oral doses of vitamin C. When these infants recovered, the blood plasma levels and urinary excretion returned to normal. When ascorbic acid was administered parenterally, a rise in blood plasma levels and urinary excretion was noted, in contradistinction to the lack of response obtained on oral administration.

A normal 10-months-old infant was placed on a metabolism frame for 3- to 4-day periods and blood plasma level,[4] urinary excretion and fecal excretion[1] of ascorbic acid were determined. As will be noted in Table I, even with the oral administration of large amounts of vitamin C supplement,* the daily excretion of vitamin C in the stools was under 4 mg. During this control period the infant averaged from one to 2 stools daily, and these were of a formed character. The administration of 4 g of magnesium sulphate dissolved in water and orally administered was followed by semi-liquid stools and a ten-fold increase in the fecal excretion of ascorbic acid.

A number of infants suffering with acute, non-specific diarrhea

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[1] Chinn, Herman, and Farmer, Chester J., Proc. Soc. Exp. Biol. and Med., 1939, **41**, 561.

[2] Abt, Arthur F., and Farmer, Chester J., *J. A. M. A.*, 1938, **111**, 1555.

[3] Meyer, L. F., and Robinson, P., *Ann. Pœdiat.*, 1939, **152**, 283.

[4] Farmer, C. J., and Abt, A. F., Proc. Soc. Exp. Biol. and Med., 1936, **34**, 146.

* We are indebted to Merck and Co., Inc., Rahway, N. J., for supplying the ascorbic acid (Cebione) used in this investigation.

We are indebted to Dr. David Cohn, Director of the Chemistry Department, Nelson Morris Research Institute, for providing laboratory space.

TABLE I.
Normal Blood Plasma Levels, Urinary and Fecal Excretions and Effect of Catharsis After Oral Ingestion of Ascorbic Acid.
Subject: E. B., age 1 yr, normal infant.

Date	Additional supplement, mg ascorbic acid*	Plasma ascorbic acid, mg %	Urinary excretion ascorbic acid, mg	Fecal excretion		No. of stools daily
				Moist wt, g	Ascorbic acid, mg	
7- 9	300					
10	300					
11	300	1.12	41.58	35	1.43	1
12	300	1.68	74.66	12	2.00	2
13	300	1.33	58.24	33	2.41	2
****						
18	300	0.67	15.16	14	0.31	1
19	300	0.71	25.63	19	2.38	2
20	300	1.26	32.88	20	2.55	2
21	0	1.75	67.68	17	3.76	2
****						
23	300					
24	300	1.22	lost	43	3.70	1
25	300	2.04	61.26	19	1.64	2
26	300	2.37	247.76	24	3.92	1
27	0	2.31	192.07	18	3.90	1
****						
8- 1	300	1.13	60.03	16	2.56	1
2	0	1.07	lost	12	2.02	1
3	300					
	plus 4 g $MgSO_4$	1.02	lost	56	1.98	2
4	0	1.33	70.00	107	22.27	2 liquid
****						
11	0	0.75	lost	32.5	1.62	1

* The infant was on a mixed dietary containing approximately 50 mg ascorbic acid daily.
**** Periods off metabolism frame. No supplement of ascorbic acid.

were placed upon a metabolism frame and blood plasma level, urinary excretion and fecal excretion of ascorbic acid were studied. The oral administration of large amounts of vitamin C was followed by a

TABLE II.
Influence of Diarrhea on Orally Ingested Ascorbic Acid, Blood Plama Level and Fecal Excretions.
Subject: M. S., age 2½ months, severe diarrhea and dehydration.

Date	Additional supplement, mg ascorbic acid*	Plasma ascorbic acid, mg %	Urinary excretion ascorbic acid, mg	Fecal excretion		Type of stools daily
				Moist wt, g	Ascorbic acid, mg	
9- 8	0	.23	0.84	204	1.76	liquid
9	200	.24	0.89	196	1.64	”
10	200	.26	1.10	410	28.25	”
11	200	.29	2.92	297	52.40	”
12	0	.26	1.89	285	34.35	semi-formed

* Dietary consisted of protein milk formula.

greatly increased fecal excretion in these infants suffering with diarrhea; Table II is an example of a 2½-month-old infant with a severe diarrhea and dehydration. Blood plasma levels and urinary excretion remained low, as noted by Meyer and Robinson.[3]

Summary. Ascorbic acid is excreted in small amounts in the stools of the normal infant studied. Large amounts of orally administered ascorbic acid are excreted in the stools of infants following catharsis and during acute diarrhea. The increased fecal excretion of orally administered ascorbic acid during acute diarrhea in the infant points to its failure of absorption in the intestinal tract, and explains the low blood plasma values and low urinary excretion.

11082 P

Does Alcohol Stimulate Gastric Secretion by Liberating Histamine?

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The stimulation of gastric secretion by alcohol is similar to the stimulation by histamine in a number of ways. They both stimulate the secretion of a juice that is highly acid but relatively low in peptic power.[1] The stimulating effect of alcohol, like that of histamine, is much more resistant to the inhibitory effect of atropine than is the usual meal stimulus.[2, 3] Similarly the stimulating effect of alcohol, like that of histamine, is more resistant to the inhibitory effect of fat feeding than is the usual meal stimulus.[3] Such similarities suggest the possibility that these two substances, alcohol and histamine, act upon the stomach by a similar mechanism. There is no known reason to suppose that histamine acts through the mediation of alcohol, while there are some reasons to suppose that alcohol may act through the mediation of histamine. In the first place the general nature of the pharmacological action of alcohol is that of a depressant, so that a bona fide stimulation of gastric secretion is not consistent with its usual effects. In the second place a large variety of

1 Kreuger, L., and MacIntosh, F. C., *Am. J. Dig. Dis.*, 1937, **4**, 104.

2 Gray, J. S., *Am. J. Physiol.*, 1937, **120**, 657.

3 Gray, J. S., and Bachrach, W. H., PROC. SOC. EXP. BIOL. AND MED., in press.

substances, such as ether, chloroform, methyl dichloride, glycerol, saponin,[4, 5, 6] etc., have the property of stimulating gastric secretion when introduced into the intestine. These substances, like alcohol, have the common properties of producing hemolysis and altering cellular permeability. Of more interest, a number of them have been shown to be capable of liberating histamine from mammalian tissues.[7] That some or all of these substances may owe their ability to stimulate gastric secretion to their ability to liberate histamine may be implied from the data available. Our interest, however, was directed toward determining whether alcohol could liberate histamine from tissues so that the theoretical conception of alcohol acting by a "histaminergic" mechanism, could have a valid basis.

The isolated lungs of guinea pigs were perfused through the pulmonary artery with Sollmann-Rademaeker's solution according to the method of Feldberg and Kellaway.[8] They were rhythmically ventilated with air throughout the experiment. Two kinds of procedures were employed. Either small amounts of alcohol (5 cc) of 7, 10, 15% (by volume) were injected into the perfusion system immediately proximal to the cannula in the pulmonary artery, or the control perfusion fluid was replaced by perfusion fluids containing 2, 3, 4, 6% of alcohol. By one method the lungs were briefly exposed to alcohol of moderately high concentrations and by the other, to prolonged contact with alcohol of lower concentrations. The perfusates were tested for histamine-like activity upon the guinea pig intestine. In all but one of 10 experiments thus far performed an increase in histamine-like activity has been found in the perfusates obtained after alcohol administration by either method. That the activity of these perfusates is due to histamine is indicated by the following observations. The activity is destroyed by boiling while alkaline, but not when acid. The activity is destroyed by incubation with histaminase.* The stimulating action of the perfusate upon the guinea pig intestine is not prevented by atropine, but is prevented by arginin. While the identification of the active substance in the perfusates as histamine is not absolute, it is certainly very strong.

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4 Ivy, A. C., and McIlvain, G. B., *Am. J. Physiol.*, 1923, **67**, 124.

5 Ivy, A. C., and Javois, A. J., *Am. J. Physiol.*, 1925, **71**, 604.

6 Ivy, A. C., Lim, R. K. S., and McCarthy, J. E., *Quart. J. Exp. Physiol.*, 1925, **15**, 55.

7 Kellaway, C. H., and Trethewie, E. R., *Abst. J. Exp. Biol. and Med. Science*, 1939, **17**, 225.

8 Feldberg, W., and Kellaway, C. H., *J. Physiol.*, 1937, **90**, 257.

* We are indebted to The Winthrop Chemical Company for the histaminase used in these experiments.

We believe, therefore, that these observations, in conjunction with present known facts regarding the stimulating action of alcohol upon gastric secretion, warrant the theory that alcohol has a "histaminergic" action and that its stimulating action upon gastric secretion depends upon this mechanism.

11083

Peptone Shock in Fetal Dogs and its Significance in the Metabolism of Histamine.

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It has previously been shown[1, 2] that "peptone" shock in dogs, which is produced by the intravenous injection of proteoses, is accompanied by and due to the liberation of histamine from the tissues of the injected animals. The source of, and the reasons for, the storage of histamine in the tissues are only incompletely understood. It seemed, therefore, of interest to determine whether histamine is present in the tissues of fetal animals, and if it is present, to determine whether it can readily be liberated with resulting shock reactions such as occur in adult animals. The studies of Code[3] indicate that the traces of histamine which are normally present in the blood of dogs occur in the cellular elements and not in the plasma. This would imply that the histamine normally circulating in the blood of a pregnant dog would not be accessible to the fetus. As there is no opportunity for the formation of histamine within the fetus by bacterial decomposition of histidine in the fetal intestine, it may be presumed that any histamine occurring in fetal tissues has been produced by local mechanisms such as histidase.[4]

The skeletal muscle and liver of a 200 g fetus, obtained by Caesarian section, were assayed for histamine by the method of Best[5] and Best and McHenry.[6] The skeletal muscle assayed less than 0.1

1 Dragstedt, C. A., and Mead, F. B., *J. Pharm. and Exp. Ther.*, 1937, **59**, 429.
2 Dragstedt, C. A., and Mead, F. B., *J. Pharm. and Exp. Ther.*, 1938, **63**, 400.
3 Code, C. F., *J. Physiol.*, 1937, **90**, 349.
4 Holtz, P., and Heise, R., *Archiv. f. exp. Path. u. Pharm.*, 1937, **186**, 377.
5 Best, C. H., *J. Physiol.*, 1929, **67**, 256.
6 Best, C. H., and McHenry, E. W., *J. Physiol.*, 1930, **70**, 349.

mg histamine base per kilo of tissue. It is, therefore, somewhat doubtful whether any histamine was present. The liver assayed 5.0 mg of histamine base per kilo of tissue.

Three 400 g fetuses, obtained at term, were anesthetized with ether and arranged for the recording of the carotid blood-pressure. Two cc per kilo of peptone solution were injected intravenously in each case, and the resulting reactions recorded. The peptone solution used was a 10% solution of Bacto-Protone-Difco, which had previously been acidified, shaken with permutit, filtered, and then neutralized. Such solutions contain negligible quantities of histamine, are rich in proteose and have been found very satisfactory.[2] A severe reaction occurred in each instance. The reaction was fatal in one, and probably would have been fatal in the others, although in these any possible recovery was prevented by bleeding them to death so that histamine determinations of the blood could be made. The blood was incoagulable in each instance. The plasma in each instance had a histamine activity equivalent to 0.6 gamma histamine base per cc when tested on the etherized and atropinized cat.

Discussion. Histamine occurs in the liver, if not in the skeletal muscle of the dog fetus. The quantity appears to be less than that occurring in adult dogs, although more information is necessary to establish the average relationship. Peptone shock can occur in the dog fetus and, as is the case in the adult dog, it is associated with the liberation of histamine. These findings would seem to have a bearing on the question of the metabolism of histamine, indicating that an appreciable part, at least, of the histamine store in the tissues owes its existence to other than bacterial production.

11084

Toxic and Therapeutic Response of Blood and Bone Marrow to Sulfanilamide.

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Sulfanilamide may produce an acute hemolytic anemia, which comes on rapidly after therapy and may terminate fatally. More commonly, however, the drug causes a mild type of anemia which is

familiar to all physicians. The acute anemia is characterized by a marked reduction in the erythrocyte count and hemoglobin, a macrocytosis, reticulocytosis, leucocytosis, and increased icterus index. Wood[1] has reported an incidence of 21 cases of acute hemolytic anemia among 522 patients treated with sulfanilamide.

Therapeutic doses of sulfanilamide were administered to 9 patients. This group consisted of 4 cases of subacute bacterial endocarditis, 2 normal patients, and one case each of streptococcus sore throat, chronic pyelitis and Hodgkin's disease. The dosage varied from 24 g to 72.3 g given over a period of 8 to 17 days. Complete haematological studies including sternal bone-marrow aspirations were made before, during and after therapy. A modification of Pontoni's[2] technic was utilized in doing the differential cell counts of the bone marrow. The mean cell diameters of the erythrocytes were computed in 4 cases. Blood sulfanilamide levels were determined according to the method modified by Marshall.[3]

It was observed that there was a definite increase in the mean corpuscular volume of the erythrocytes after the administration of sulfanilamide. The average mean corpuscular volume (Table I) of the 9 cases before therapy was 86.0 cubic microns and after, 100.2 cubic microns respectively. The increase in volume was not dependent on the dosage of sulfanilamide. The effect of sulfanilamide upon the total erythrocyte count was variable. The depression in the erythrocyte count ranged from 190,000 to 1,280,000 with an average drop of 660,000. The hemoglobin, leucocytes, reticulocytes and icterus index were not markedly altered.

With the exception of some increase in the number of stab forms, the differential white cell count remained practically unchanged. Normoblasts were not found in smears of the peripheral blood. The mean cell diameter of the erythrocyte was slightly increased. The

TABLE I.
Average Value of Nine Cases Before and After Sulfanilamide.

	Hemoglobin	Erythrocytes	Leucocytes	Hematocrit	Mean corpuscular vol.	Reticulocytes %	Icterus index	g sulfanilamide
Before sulfanilamide	11.5	3,96	8,883	34.2	86.0	0.85	5.2	41
After sulfanilamide	10.3	3,30	7,816	33.1	100.2	1.14	6.1	

[1] Wood, W. B., *J. A. M. A.*, 1938, **111**, 1916.

[2] Pontoni, L., *Hæmatologica*, 1936, **17**, 833.

[3] Marshall, E. K., *J. Biol. Chem.*, 1937, **122**, 263.

free sulfanilamide level of the blood varied from 2.1 mg to 12.5 mg per 100 cc of blood with an average value of 6.2 mg per 100 cc of blood.

Therapeutic doses of sulfanilamide produce a moderate normoblastic bone marrow hyperplasia with most of these cells at the orthochromatic stage. The myeloid elements and megakaryocytes showed no conspicuous change. The differential cell count of the bone marrow showed an average myeloid-erythroid percentage of 69.5% to 30.5% before therapy, and a value of 46.2% to 53.8% after therapy. There is no relationship between the dosage of the drug and the normoblastic reaction of the bone marrow.

The toxic effect of sulfanilamide was observed in one case (Table II). It will be noted that an acute hemolytic anemia developed after the administration of 15 g over a period of 8 days. This was manifested by a marked depression of the hemoglobin and erythrocyte count, pronounced leucocytosis, macrocytosis, reticulocytosis, and elevated icterus index.

TABLE II.
Case of Acute Hemolytic Anemia.

	Hemoglobin	Erythrocytes	Leucocytes	Hematocrit	Mean corpuscular vol.	Reticulocytes %	Icterus index	g sulfanilamide
Before	9.1	3,50	14,500	32.0	91.4	0.9	5.0	
								15 in 8 days
After	5.25	1,66	57,800	18.0	108.4	58.0	15.0	

A moderate shift to the left of the Schilling Index was found in the differential cell count of the peripheral blood. The bone marrow showed a marked normoblastic and pronormoblastic reaction with most of the normoblasts of the basophilic type. The differential cell count showed a myeloid-erythroid percentage of 71% to 29% before therapy, and a value of 26.5% to 73.5% after therapy. The megakaryocytes were not affected.

Machella and Higgins[4] have recently demonstrated a marked anemia with increase in the volume of the red cells in white rats following the administration of 1 g of sulfanilamide per kilo of body weight. Using therapeutic doses of the drug in man, our results were quite similar. Continuation of sulfanilamide therapy after the development of macrocytosis did not induce an acute hemolytic process. An increase in the volume of the red cells was usually found to occur about the seventh day. The acute hemolytic anemia pro-

4 Machella, T. E., and Higgins, G. M., *Am. J. Med. Sci.*, 1939, **198**, 804.

duced by sulfanilamide differs from the macrocytic anemia just mentioned in that it develops between the third and sixth days of therapy and is rapidly progressive in degree. By virtue of its rapid onset after the use of therapeutic doses, it is reasonable to believe that this form of anemia is due to an idiosyncrasy or susceptibility to the drug, rather than to the administration of toxic doses.

The macrocytic anemia which commonly follows treatment with sulfanilamide can be ascribed either to the development of an acute hepatitis or the direct effect of the drug upon the bone marrow. Due to the relatively short interval between the onset of therapy and the macrocytosis, it is not likely that the anemia is induced by liver changes. On the contrary, evidence of the action of sulfanilamide on bone marrow is demonstrated by its normoblastic response.

Conclusion. Therapeutic doses of sulfanilamide commonly produce macrocytic anemia and a normoblastic bone-marrow reaction. In the acute hemolytic anemia the bone marrow shows a more marked normoblastic reaction with predominance of young forms.

11085

Estrogen-Induced Hypospadias in the Female Rat.*

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Normal development of the external genitalia of the female rat can be grossly modified by treatment with estrogens, either antepartum or immediately postpartum.[1-4]

The normal female rat has a clitorine urethra with the urinary meatus located at the tip of the clitoris (the word "clitoris" is used to indicate the female phallus and not just the glans clitoridis). The proximal portion of the clitorine urethra is anatomically a true

* Supported in part by a grant from the Josiah Macy, Jr., Foundation. The alpha estradiol (Ovocylin) and estradiol dipropionate (Di-Ovocylin) were furnished through the courtesy of Ciba Pharmaceutical Products, Inc.

1 Hain, A. M., *Edinburgh Med. J.*, 1935, **42**, 101.

2 Greene, R. R., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 503.

3 Greene, R. R., and Ivy, A. C., *Science*, 1937, **86**, 200.

4 Turner, C. D., and Burkhardt, W. L., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 267.

urethra, while the distal portion of the functional urethra is formed by the preputium which surrounds and extends beyond the end of the glans clitoridis.

In the estrogen-modified animals no such clitorine urethra is present. The urethral meatus is located in an abnormal position, at the base of the phallus. The phallus itself is cleft on the caudal surface. Its component structures are therefore exposed.

A more extensive abnormality has been produced in an unreported group of 10 adult females. These animals received no direct treatment. However, their mothers received very large doses of estrogens during pregnancy. The mothers of 9 animals were given estradiol dipropionate in dosages varying from 3.0 to 50.0 mg between the 12th or 13th to the 17th or 19th days of pregnancy. The mother of the 10th animal received 5.5 mg of alpha estradiol between the 13th and 20th days of pregnancy. The clitorides of these animals presented the anomalous conditions already described. In addition, these animals had no discrete vaginal orifice. The small orifice located at the base of the cleft phallus, on investigation, proved to be a common opening for both urethra and vagina. These two structures joined to form a common canal 3 to 4 mm proximal to the external orifice.

Observations on normal development have been presented previously in conjunction with studies on the effects of androgens on sexual development.[5] In order to interpret the abnormality noted above it is necessary to review briefly the normal embryonic and postnatal development of the female. In the female rat the caudal portion of the vagina is formed during embryonic development by a longitudinal fission of the urogenital sinus into a ventral portion (urethra) and a dorsal portion (lower vagina). This fission, studied by consecutive wax reconstructions, proceeds caudalward, but even at birth is not quite complete. The most caudal portion of the vagina is still connected to the urethra by a bridge of epithelial cells and is not patent. The urinary meatus at birth is located at the caudal base of the phallus and represents the still unclosed primary urogenital ostium. Late in embryonic development the median urethral groove is formed on the caudal face of the phallus. At birth this median groove is continuous with the urinary meatus at the base of the phallus. Both are macroscopically visible. During normal postnatal development this urethral groove is roofed over by fusion of the 2 halves of the preputium which meet in the midline of the caudal surface of the phallus. As a result of this fusion the urethral groove

[5] Greene, R. R., Burrill, M. W., and Ivy, A. C., *Am. J. Anat.*, 1939, **65**, 415.

is transformed into the clitorine urethra and the urogenital ostium is closed so that the urinary meatus is transferred from the base of the phallus to its tip. The male rat presents a more advanced stage of development at birth. With a few exceptions the penile urethra is already completely formed and the primary urogenital ostium is closed at birth.

There is an obvious similarity between the conditions of the external genitalia in the estrogen-modified females and the conditions in the embryonic states. In these modified adult females, as in the 21-day-old fetus, the more caudal portions of the vagina and urethra have not separated. Some development beyond the fetal stage has taken place in that the vagina is completely canalized, but the last 3 to 4 mm of both urethra and vagina are still represented by their normal precursor, the urogenital sinus. The urethral meatus in these animals is still situated as it is in the fetal and also the newborn state, in the position of the primary urogenital ostium, *i. e.,* at the base of the cleft phallus.

The phallus of these females remains in the cleft condition due to the fact that the preputial folds have failed to fuse and thus no clitorine urethra is formed. Subsequent growth of other parts of the phallus concomitant with growth of the animal to the adult state tends to exaggerate the defect. What appears as a groove in the fetus or newborn animal becomes a wide open cleft in the adult, exposing the glans clitoridis and also certain other homologues of the male cavernous structures which are normally present in newborn and adult female rats.

In the less modified animals previously reported by various workers, the degree of developmental arrest is less extensive and resembles more the conditions in the normal newborn female rather than the conditions in the fetus. Some degree of development occurs in that the vagina and urethra are no longer contiguous as they are normally at birth. The vagina therefore has an external orifice which is separate from the urethral orifice. The latter, however, is maintained in the location of the primary urogenital ostium at the base of the phallus.

The chief distinction between the two types of defects is that in the more highly modified animals the development of the caudal portion of the vagina is completely inhibited so that, in effect, the lower portion of the urogenital sinus is retained as in the fetal state, whereas in the less highly modified animals vaginal development continues to a point where the vagina has an external opening separate from that of the urethra. In both types, however, the urinary orifice is located in an abnormal position, at the base of the cleft phallus.

These genital defects, which are produced by estrogenic treatment, therefore, are due to inhibition of normal development.

The explanation of this defect provided by Turner and Burkhardt[4] obviously neglects consideration of embryonic and normal postnatal development. It is apparently based on gross observations of the abnormality in 6 out of 16 treated animals and on comparison with the normal fully developed female. These authors believe that the extensive fissure in the clitorine prominence apparently results from more extensive cleavage of the preputial fold than occurs normally. This is very unlikely inasmuch as no cleavage of the preputial fold normally occurs. The process involved is definitely an inhibition of the normal fusion of the two halves of the preputium. The "erectile" bodies noted by Turner and Burkhardt are merely the homologues of the male cavernous structures which are normally present in the female. The lack of development and fusion of the preputial folds has left these structures exposed.

The defect is not due to "resorption of a portion of tissue which developmentally forms the anterior wall of the urogenital sinus," as Hain has suggested[1] because there is no tissue covering the urethral groove to be resorbed.

Turner and Burkhardt have objected to the application of the term "hypospadias" to the genital abnormality under consideration. The normal female rat has a clitorine urethra while these modified animals do not. Instead, the urinary meatus is located at the base of the phallus in the position of the primary urogenital ostium. The term "hypospadias" therefore seems to be applicable, since it is anatomically descriptive of the defect. This same hypospadias has been produced in male rats by the administration of very high doses of estrogens to the pregnant mothers. In the males the defect is also due to inhibition of normal development.[6]

Summary. The administration of very large doses of estrogens to pregnant rats has caused a permanent hypospadias and lack of development of the most caudal portion of the vagina, in the female offspring. The abnormality represents an arrest of development so that conditions similar to those found in the 21-day fetus are retained.

[6] Greene, R. R., Burrill, M. W., and Ivy, A. C., unpublished data.

11086

Effects of Atropine and Fat on Gastric Secretion Stimulated by Alcohol.

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Although dilute alcohol has become a popular gastric test meal, little is known about its mode of action or the possible influence of other drugs upon its action. Local contact with the gastric mucosa appears not to be essential for its action since alcohol has been reported to be an effective stimulus for the secretion of acid gastric juice when introduced into the rectum (Babkin[1]) or into the small intestine,[2, 3] or even intravenously.[4, 5] Neither section of the vagi[6] nor the sympathetics[7] interferes with its action on the gastric glands. Orbeli[6] has stated that atropine in very large doses (5 mg) abolished the effect of alcohol in one dog. Kreuger and MacIntosh[8] have more recently stated that large doses (0.3 mg per kg) of atropine completely inhibited the secretion of gastric juice in response to alcohol; smaller dose, however, served merely to diminish the response. These results suggest that alcohol resembles histamine, which has been shown to be much more resistant to the inhibitory effect of atropine than is the usual meal stimulus.[9] In order to investigate this possibility further, the effect of atropine on the gastric secretory response to alcohol and to a meal has been determined in Pavlov pouch dogs.

Methods. Three dogs with the Pavlov type of gastric pouch were used. In one series of experiments 100 g of beef hearts ground and brought to a boil in 100 cc of water were fed to the dogs either alone, or with 50 cc of olive oil, or with 1 mg of atropine sulfate adminis-

1 Babkin, B. P., *Die Aussere Sekretion der Verdauungsdrusen*, Julius Springer, Berlin, 1928.

2 Chittenden, R. H., Mendel, L. B., and Jackson, H. C., *Am. J. Physiol.*, 1898, **1**, 164.

3 Ivy, A. C., and McIlvain, G. B., *Am. J. Physiol.*, 1923, **67**, 124.

4 Petrovitch, A., and Bokanowa, E., *C. R. Soc. Biol.*, 1929, **102**, 633.

5 Newman, H. W., and Mehrtens, H. G., Proc. Soc. Exp. Biol. and Med., 1932, **30**, 145.

6 Orbeli, L. A., *Arch. des. Sci. biol.*, 1906, **12**, 71.

7 Ishido, B., *Biochem. Z.*, 1922, **130**, 151.

8 Kreuger, L., and MacIntosh, F. C., *Am. J. Dig. Dis.*, 1937, **4**, 104.

9 Gray, J. S., *Am. J. Physiol.*, 1937, **120**, 657.

TABLE I.
Effects of Atropine and Olive Oil on Gastric Secretion.

	Dog No. 1					Dog No. 2					Dog No. 3				
	No. of Tests	Vol. cc	Free acid mg	Total acid mg	% Inhib. of free acid	No. of Tests	Vol. cc	Free acid mg	Total acid mg	% Inhib. of free acid	No. of Tests	Vol. cc	Free acid mg	Total acid mg	% Inhib. of free acid
100 g beef heart	10	41.1	109.6	144.2		11	26.7	57.8	75.1						
" " " " —1 mg atropine	2	12.0	0	8.2	100	3	6.4	0	0.8	100					
" " " " —50 cc olive oil	6	19.1	4.4	39.1	96	4	7.2	1.2	4.9	98					
120 cc of 7% alcohol	6	15.0	18.5	31.5		7	14.8	22.8	31.5		8	14.5	14.7	27.8	
" " " " " —1 mg atropine	6	8.2	5.5	10.6	66	5	8.5	5.8	12.6	75	4	10.4	10.4	18.3	29
" " " " " —50 cc olive oil	6	11.8	18.2	25.8	18	6	13.8	15.3	25.4	19					

tered subcutaneously. In a second series of experiments the meat meal was replaced by 120 cc of 7% alcohol administered by stomach tube. In all cases the gastric juice was collected over a 3-hour period and titrated for free acid (Topfer's reagent) and total acid (phenolphthalein).

Results. The results, in the form of averages for the different experiments are presented in Table I. It can be seen that 1 mg of atropine sulfate completely abolished the gastric secretory response to the meat meal, but only partially reduced the response to 7% alcohol. Similarly, the alcohol stimulus proved to be more resistant than the meat meal to the inhibitory action of olive oil.

The available evidence suggests that a close resemblance exists between the actions of alcohol and histamine on the gastric glands. The action of both is resistant to the inhibitory action of atropine and fat, and Kreuger and MacIntosh[8] have reported that both stimulate the production of a juice of high acidity and low pepsin concentration.

Conclusions. In dogs with Pavlov pouches the gastric secretory response to dilute alcohol is resistant to the inhibitory effects of atropine or fat.

11087

Photoelectric Study of Liebermann-Burchard Reaction and Its Significance in Determination of Cholesterol.

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The common colorimetric method for the determination of cholesterol is based upon the Liebermann-Burchard[1] reaction, in which acetic anhydride and concentrated H_2SO_4 are added to a dilute solution of cholesterol in chloroform. The color produced is at first blue, then becomes green and finally, on long standing, a yellow-brown. This reaction, in spite of its apparent simplicity, has proved difficult to control, for the intensity as well as the shade of the color is markedly influenced by small differences in the concentrations of the reagents, the presence of traces of water or other impurities, time, and temperature. The many previous efforts to control these factors

[1] Liebermann, C., *Ber. Deut. Chem. Ges.*, 1885, **18**, 1803.

by modification of the technique of Autenrieth and Funk[2] and of Bloor[3] have been for the most part empirical. A study of the reaction, however, with the aid of the photoelectric colorimeter and varying filters gives a clue to the nature of the reaction and the principles that must be followed for exact, duplicable, colorimetric analysis of cholesterol.

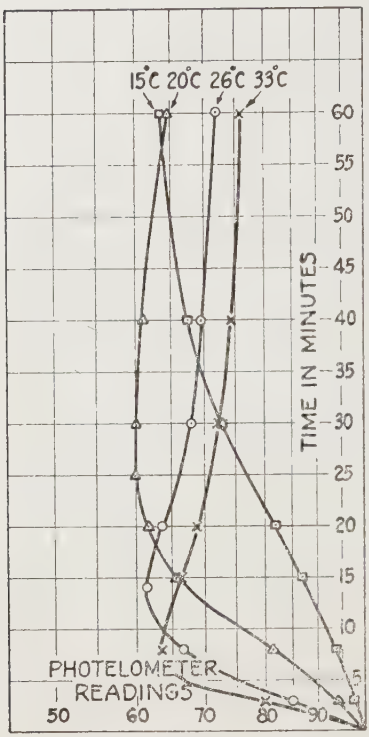
A mixture of 15 cc of acetic anhydride, 1 cc of concentrated H_2SO_4 and 24 cc of chloroform, C.P. was used as the reagent. This mixture was prepared immediately before using, since it decomposes on standing for more than an hour. Five cc were added with thorough mixing to 5 cc of a chloroform solution containing 0.48 mg of cholesterol in a glass-stoppered cylinder. The vessel was immediately placed in a water bath maintained at the proper temperature. This procedure, which was also followed in the routine analysis of cholesterol, eliminated errors due to inaccuracy of measurements of small quantities of acetic anhydride and H_2SO_4 and to irregular initial temperatures produced by direct addition of H_2SO_4, and allowed accurate measurement of the effect of time and temperature. Readings were made with a photoelectric colorimeter with an orange filter with a maximal transmission at 620 mμ, or with a blue filter with a maximal transmission at 430 mμ.* The curves obtained were plotted on semi-logarithmic paper, since the concentration of the light-absorbing substance is proportional to the negative logarithm of the reading.

At 26°C, with an orange filter, the absorption progresses to a maximum in 13 minutes, then slowly recedes and approaches zero (Fig. 1). With a blue filter, absorption increases continuously with time, shows no maximum in an hour, but at that time is approaching a constant value (Fig. 2). At higher temperatures with the orange filter, a maximal absorption is reached earlier, but the value is less. At temperatures lower than 26°C, a maximal absorption is more delayed, but when reached has a higher value and is maintained for a longer time before recession occurs. At 15°C, a maximal absorption is not obtained in an hour. Further readings at 15°C indicate that the reagent decomposes before a true maximum is reached. With a blue filter, curves at various temperatures are similar to each other, but the absorption at 60 minutes is proportional to the temperature (Fig. 2). At 10 minutes, the effect of temperature change

2 Autenrieth, W., and Funk, A., *Münch. Med. Woch.*, 1913, **60**, 1243.

3 Bloor, W. R., *J. Biol. Chem.*, 1915, **23**, 317.

* The Cenco-Sheard-Sanford Photelometer, manufactured by the Central Scientific Company, was used in these experiments.



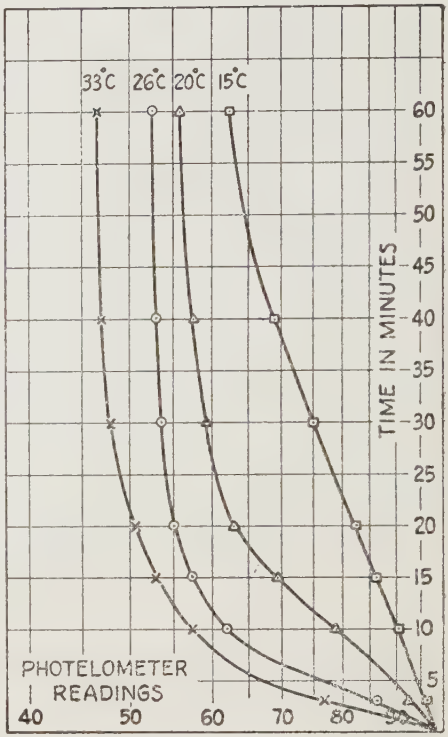


FIG. 1 (Left)
The relationship between light-absorption with an orange filter and the time of the development of the color, at various temperatures. A reading of 100 indicates no absorption.

FIG. 2 (Right)
The relationship between light-absorption and time at various temperatures, with a blue filter.

upon the reading is much greater for both filters in the lower temperature range than in the higher.

These results indicate that the Liebermann-Burchard reaction consists of 2 phases: A, the production of a blue substance which has absorption bands both in the region of the blue and of the orange; B, the decomposition of this blue substance, as soon as it is formed, into a yellow substance which has an absorption band only in the blue. Reaction B is affected by increased temperature more than is A. Therefore, at higher temperatures, the point is reached sooner where the decomposition of the blue substance is as fast as its formation, and the sooner this point is reached, the less there is of the blue substance.

Spectrophotometric examination of the colored solution with the

Cenco-Sheard Spectrophotelometer confirmed these findings.† There are 2 broad absorption bands, one between 600 and 660 mμ with a maximum at 630, and another between 380 and 440 mμ with a maximum at 420 mμ. As the solution changed from blue to green to yellow-brown, the absorption band in the orange-red became more shallow while that in the blue became deeper.

It is obvious that these facts must be considered in applying the Liebermann-Burchard reaction to the analysis of cholesterol. All the factors which determine the intensity and shade of the color, particularly time and temperature must be controlled. This is especially true of photoelectric methods, for here analyses of unknown and standard solutions are not made simultaneously. In the photoelectric method, either an orange or a blue filter may be used, but the former is preferable since the reading can be made at a maximal value after a short time. Besides, colored impurities in the reagents give little blank with an orange filter, but may show appreciable and variable blanks with a blue filter. Since the time of the reading can be fixed more readily than the temperature, it is best to make the readings with the orange filter at exactly 10 minutes at a temperature of 26°C or slightly above, for at this time, and this temperature range, there is the least effect of change of temperature upon the reading.

In the actual determination of cholesterol in blood, 0.2 cc of serum (or whole blood) are allowed to dry on a filter paper disc and then extracted with about 4 cc boiling chloroform for 60 minutes in a Leiboff[4] extraction tube. The cooled chloroform solution is then quantitatively transferred to a 10 cc glass stoppered cylinder, made up to 5 cc with chloroform, and treated with 5 cc of the freshly prepared reagent. The cylinder is placed in a water bath kept between

TABLE I.
Accuracy of Determination of Cholesterol.
The results are expressed as mg per 100 cc.

Sample	No. of Determinations	Concentration	Added Cholesterol	Total Cholesterol	
				Calculated	Found
Cholesterol solutions					
240 mg per 100 cc	8	240 ± 3			
160 mg per 100 cc	8	160 ± 2			
Serum	8	178 ± 5	80	258	252
Serum	8	184 ± 4	100	284	294
Whole blood	8	148 ± 5	160	308	310

† The author is indebted to Dr. M. N. States, of the Central Scientific Company, for his assistance in the spectrophotometric study.

[4] Leiboff, S. L., *J. Biol. Chem.*, 1924, **61**, 177.

27°C and 26°C for 9 minutes. The colored liquid is then transferred to a fused glass absorption cell and read in the photelometer with an orange filter at exactly 10 minutes. The value is read from a calibration curve obtained from the analyses of standard cholesterol solutions in chloroform made exactly in the same manner. The curve plotted on semi-logarithmic paper is a straight line.

The accuracy and duplicability of the method is attested to by the findings in Table I. Added cholesterol was recovered with the same degree of accuracy.

11088

Effect of Adrenocorticotropic Hormone in 4-Day-Old Rats.*

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Adrenocorticotropic hormone (A-C-T) has been assayed in this laboratory using the 21-23-day-old male rat.[1] This test requires relatively large amounts of A-C-T. For this reason the response of 4-day-old rats to A-C-T has been studied with the hope of finding them to be more sensitive.

On the day of birth, rats were grouped into litters of 8, containing both males and females. Beginning on the 4th day postpartum, the rats in each litter were injected intraperitoneally with 0.1 ml of A-C-T preparations (previously assayed in 21-23-day-old rats)

TABLE I.
Effect of A-C-T on 4-day-old Rats.

No. of rats	Dose units	Avg body wt		Avg wt of adrenals mg	Avg wt of thymus mg
		Init. grams	Final grams		
16	0.05	9.5	12	3.7	10.5
16	0.10	9.6	14.2	4.0	9.5
16	0.15	9.5	11.5	4.3	5.1
16	0.25	9.0	11.2	4.4	5.3
16	0.50	—	10	6.1	2.5
40 controls		9.8	14.0	2.4	20.9

* Aided by grants from the Board of Research of the University of California and Rockefeller Foundation of New York City. Assistance was rendered by the Federal Works Progress Administration, Project O.P. 65-1-08-62 Unit A-5.

[1] Moon, H. D., PROC. SOC. EXP. BIOL. AND MED., 1937, **35**, 649.

daily for 3 days. The litter was then sacrificed 24 hours after the last injection, the adrenals and thymuses dissected out, fixed, and then weighed. The adrenals were weighed together and the thymuses were weighed separately unless the atrophy was nearly complete. In some cases only 4 of the littermates were injected and 4 kept as controls. Table I shows the effects of A-C-T on adrenals and thymuses of 4-day-old rats.

Mammotropic† and growth‡ preparations as well as inactivated A-C-T preparations were given to the 4-day-old rats. Thymic atrophy was not produced by these preparations.

TABLE II.
Effect of Mammotropic and Growth on 4-day-old Rats.

No. of rats	Extract	Total dose units	Avg adrenal wt, mg	Avg thymus wt, mg
16	Mammotropin	50[2]	2.6	19.7
16	Growth hormone	5§	2.6	24.6
16	" "	10	2.9	28.2

[2] Lyons, W. R., PROC. SOC. EXP. BIOL. AND MED., 1937, **35**, 645.

§ 1 unit of Growth Hormone: the daily amount required to cause plateaued female rats to gain 2 g per day over a period of 20 days.

Celloidin sections were cut of the adrenals and thymuses, and stained with hematoxylin and eosin. Microscopic examination revealed that the adrenals of the rats injected with 0.05 units showed definite evidences of stimulation.‖ When compared to the controls, the cells throughout the adrenal cortex were larger; there was an increase in the number of mitoses; there was vascular congestion in the cords of cells in the zona fasciculata; there was an increase in the amount of fat vacuoles. These changes were more marked in the adrenals of rats injected with larger amounts of A-C-T.

The atrophic thymuses of rats treated with A-C-T showed a loss of the lymphocytes. There appeared to be a *relative* increase in number of epithelial cells, although there was no evidence of actual hyperplasia of the epithelial elements of the thymus.

It is to be noted that even at the lowest level of A-C-T administered (0.05 units) there was gravimetric (Table I) as well as histologic evidence of stimulation of the adrenal cortex. Thus 0.05 unit will cause a 50% increase in the adrenal weights of 4-day-old rats,

† Prepared and kindly supplied by Dr. W. R. Lyons of this laboratory.

‡ Prepared and kindly supplied by Mr. Donald Meamber of this laboratory.

‖ With more active A-C-T preparations, this response was obtained with as little as 0.2 mg of total solids. These preparations were obtained by repeated solution and precipitation from acid acetone solutions and isoelectric precipitations from aqueous solutions as described in the method first published (*Cf.* Ref. 1).

whereas it requires one unit to cause a 50% increase in the adrenal weights of 21-day-old rats. We believe that 4-day-old rats afford a more satisfactory method of assay for A-C-T because of greater sensitivity.

Summary. The response of 4-day-old rats to various anterior pituitary extracts has been described. It was noted that 4-day-old rats responded with adrenal hypertrophy and thymic atrophy to much smaller amounts of A-C-T than 21-23-day-old rats. Mammotropic and growth preparations did not cause adrenal hypertrophy and thymic atrophy. It is concluded that the 4-day-old rats will be a more sensitive test animal for A-C-T preparations.

11089

Effect of Adrenocorticotropic Hormone on the Thymus of Rats.*

ROBERT H. CREDE AND HENRY D. MOON. (Introduced by H. M. Evans.)

From the Institute of Experimental Biology and the Department of Anatomy, University of California, Berkeley.

It was reported in earlier papers that the administration of adrenocorticotropic hormone (A-C-T) to immature rats resulted in adrenal hypertrophy[1, 2] and thymic atrophy.[2, 3] It has also been noted that large amounts of A-C-T will also cause a depression of somatic growth, whereas the viscera continue to grow. These findings have been studied considerably more in detail in this laboratory.†

A-C-T, prepared and assayed by the method previously published,[1] was given at various levels to 21-23-day-old normal male rats in 3 daily injections. The rats were autopsied 24 hours after the last injection. It was noted that the thymic atrophy was proportional to the amount of A-C-T administered as seen in Table I.

Atrophy of the thymus following the administration of the gonadotropic hormone of pregnant mare serum was reported by Evans and

* Aided by grants from the Board of Research of the University of California and Rockefeller Foundation of New York City. Assistance was rendered by the Federal Works Progress Administration, Project O.P. 65-1-08-62 Unit A-5.

1 Moon, H. D., PROC. SOC. EXP. BIOL. AND MED., 1937, **35**, 649.

2 Moon, H. D., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 34.

3 Evans, H. M., Moon, H. D., Simpson, M. E., and Lyons, W. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 419

† Results on the inhibition of growth to be published later.

TABLE I.
Effect of A-C-T on Normal Immature Male Rats.

No. of rats	Age at autopsy, days	A-C-T units	Length of treatment, days	Adrenals, mg	Thymus, mg
80	26-28	Controls	3	16.9	174
5	26	1/8	3	18.2	146
5	26	1/4	3	18.6	133
5	26	1/2	3	19.6	116
10	26-27	1	3	24.1	78
6	27	2	3	33.1	33
6	27	4	3	49.9	19

Simpson.[4] This atrophy was mediated through the gonads as shown by the effectiveness of the sex hormones in causing thymic atrophy.[5]

In order to rule out the possibility of adrenocorticotropic hormone causing thymic atrophy through the gonads, extracts were given to castrate rats. Table II shows that A-C-T caused just as marked atrophy of the thymus in castrate rats as in normal rats.

TABLE II.
Effect of A-C-T in Castrate Rats.

No. of rats	Age at autopsy, days	A-C-T units	Length of treatment, days	Avg wt of adrenals, mg	Avg wt of thymus, mg
6 males	26	1.0	3	26.0	96.0*
6 "	25	1.25	3	28.5	78.5*
12 "	26	Controls		19.0	220.0*
6 females	46	40	25	125.0	33.0
8 "	46	Controls		44.0	462.9

*Compare with Table I.

To determine whether or not the action of the A-C-T on the thymus was mediated through the adrenals, adrenalectomized animals were injected with A-C-T. Male rats were adrenalectomized when 21 to 22 days old. Injections were begun on the day following operation and given in 3 daily doses. The animals were autopsied 24 hours after the last injection. Table III shows that A-C-T had no effect on the thymus of adrenalectomized rats.

TABLE III.
Effect of A-C-T in Adrenalectomized Rats.

No. of rats	Age at operation, days	Age at autopsy, days	A-C-T units	Length of treatment, days	Thymus, mg
6 males	22	26	4	3	197
8 "	22	26	Controls	—	178

[4] Evans, H. M., and Simpson, M. E., *Anat. Rec.*, 1934, **60**, 423.

[5] Schacher, J., Browne, J. S. L., and Selye, H., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 488.

A-C-T has also been given to hypophysectomized rats at various levels to determine the amount of repair of the adrenal cortex as well as to demonstrate thymic atrophy. It was noted that when a total of 1 unit or less of A-C-T was given, that no consistent decrease in the thymus could be demonstrated although this amount of A-C-T stimulated the adrenals. However, when more than 2.5 units were given over a period of 10 days there was always marked atrophy of the thymus.

Conclusions. 1. A-C-T caused acute thymic atrophy in normal 21-23-day-old rats. 2. Castration did not prevent thymic atrophy produced with A-C-T. 3. This atrophy was observed in hypophysectomized rats although to a lesser degree. 4. A-C-T did not cause thymic atrophy in adrenalectomized rats. 5. It would seem likely that thymic atrophy produced with A-C-T is through the adrenal cortical steroids.

11090

Response of Thyroidectomized Rats to Adrenocorticotropic Hormone.*

HENRY D. MOON AND WAYNE HANSEN. (Introduced by H. M. Evans.)

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It has been reported by McQueen-Williams[1] and Emery and Winter[2] that adrenal hypertrophy could not be obtained in thyroidectomized rats with the administration of pituitary substance. This has been interpreted as evidence for a pituitary factor which acts on the adrenals through the thyroid. Recently Rosen and Marine[3] have presented data showing a decreased response of the adrenal cortex of guinea pigs to prolonged injections of an acetic acid extract of pituitary following thyroidectomy.

The evidence presented by McQueen-Williams and Winter and

* Aided by grants from the Board of Research of the University of California and Rockefeller Foundation of New York City. Assistance was rendered by the Federal Works Progress Administration, Project O.P. 65-1-08-62 Unit A-5.

1 McQueen-Williams, M., PROC. SOC. EXP. BIOL. AND MED., 1934, **32**, 296.

2 Emery, F. E., and Winter, C. A., *Anat. Rec.*, 1934, **60**, 381.

3 Rosen, S. H., and Marine, D., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 647.

Emery[4] for a directly acting adrenocorticotropic hormone is the compensatory adrenal hypertrophy after unilateral adrenalectomy in thyroidectomized rats. Recently there has been increased evidence for a directly acting adrenocorticotropic hormone. In a previous paper from this laboratory,[5] there was a footnote stating that removal of the thyroid gland did not effect the adrenal hypertrophy obtained in immature male rats treated with adrenocorticotropic hormone. Atwell[6] has reported repair of the interrenal bodies in hypophysectomized thyroidectomized tadpoles with pituitary extracts. Jores and Boecker[7] were able to obtain adrenal hypertrophy with adrenocorticotropic extracts in thyroidectomized guinea pigs.

Adrenocorticotropic hormone (A-C-T) prepared and assayed by the method described in a previous paper[4] was injected intraperitoneally (1% solution) into normal and thyroidectomized† male rats (22 to 23 days old) daily for 3 days. The animals were sacri-

TABLE I.
Normal Rats.

No. of rats	A-C-T injected, units	Wt of 2 adrenals	
		Range, mg	Avg, mg
75	0	9.9-22.0	16.7
6	0.2	15.0-23.2	18.6
6	0.4	19.0-24.3	21.5
6	0.8	19.4-25.0	22.6
6	1.2	22.0-32.0	26.0
6	2.0	30.2-36.0	33.8
6	5.0	50.0-66.2	59.4

TABLE II.
Thyroidectomized Rats.

No. of rats	A-C-T injected, units	Wt of 2 adrenals	
		Range, mg	Avg, mg
26	0	11.5-22.0	16.6
8	0.75	16.5-24.0	20.0
8	1.50	20.0-36.5	27.9
6	3.40	23.2-40.4	35.4

4 Winter, C. A., and Emery, F. E., *Am. J. Physiol.*, 1936, **116**, 164.

5 Moon, H. D., PROC. SOC. EXP. BIOL. AND MED., 1937, **35**, 649.

6 Atwell, W. J., *Am. J. Physiol.*, 1937, **118**, 452.

7 Jores, A., and Boecker, W., *Z. f. ges. Exp. Med.*, 1937, **100**, 332.

† All thyroid tissue visible under a dissecting microscope was removed. The region was dissected and examined at autopsy. Although there was the possibility of the presence of microscopic remnants of thyroid tissue, it was considered that if thyroid activity was concerned in the adrenal hypertrophy with adrenocorticotropic hormone, there would be a significant decrease in such activity after relatively complete thyroidectomy. Injections were begun on the rats on the day following thyroidectomy.

TABLE III.
Desiccated Thyroid and A-C-T in Normal Immature Male Rats.

No. of rats	Desiccated thyroid, mg	A-C-T units	Wt of 2 adrenals	
			Range, mg	Avg, mg
6	25	0	14.5-19.0	16.2
6	25	1	20.9-26.9	24.6
6	0	1	22.1-27.0	25.2

ficed on the day following the last injection. The adrenals were removed, weighed and fixed in Bouin or 10% formol.

Normal male rats were given desiccated thyroid alone and in combination with A-C-T. The desiccated thyroid was given in an aqueous suspension by stomach tube. A single administration was given and the animals autopsied on the fourth day. A-C-T injections were begun on the day that the desiccated thyroid was given, and the injections were given daily for 3 days.

The results in Table III show that the administration of desiccated thyroid produced no augmentation of the effect of A-C-T on the adrenals. The efficacy of the desiccated thyroid was indicated by the fact that all the rats given the desiccated thyroid lost from 2 to 4 g from their initial body weight (A-C-T preparations cause an inhibition of growth but not a *loss* in body weight).

Celloidin sections stained with iron haematoxylon and aniline blue, and frozen section stained with Sudan III and haematoxylin showed no significant differences between the adrenals of normal and thyroidectomized animals treated with adrenocorticotropic hormone.

Conclusions. The adrenal hypertrophy of normal and thyroidectomized rats has been shown to be directly proportional to the amount of adrenocorticotropic hormone administered. There was no evidence that the presence of the thyroid gland was necessary for the adrenal cortical hypertrophy produced by adrenocorticotropic preparations when given immediately after thyroidectomy. The administration of desiccated thyroid to normal rats did not augment the adrenal hypertrophy caused by adrenocorticotropic hormone. Further work will be necessary to determine whether or not a certain minimal amount of a thyroid principle is necessary in the production of adrenal hypertrophy.

11091 P

Rise of Blood Pressure During Ischemia of the Gravid Uterus.*

Eric Ogden, G. J. Hildebrand and Ernest W. Page. (Introduced by N. W. Shock.)

From the Division of Physiology, University of California Medical School.

To test the concept that diminished uterine blood supply might play some part in the hypertension of eclampsia[1] the carotid blood pressure was recorded in acute experiments while the aorta was partially occluded below the renal arteries.

Ten animals anesthetized with chloretone, with morphine and pentobarbital sodium, or by decerebration under ether were used. Six were pregnant (5 dogs, 1 cat); 4 were non-pregnant controls.

The carotid and femoral blood pressures were recorded kymographically and a long-handled screw clamp was adjusted around the aorta just below the renal arteries. The incision was closed with the clamp handle protruding and the animal was left undisturbed until the blood pressure was stable. The clamp was then tightened until the femoral pressure fell to about half its previous value and the carotid pressure was followed (Table I).

In the non-pregnant control animals aortic compression was not followed by any change in carotid pressure other than the immediate adjustments discussed by Brotchner.[2] Rytand,[3] Brotchner,[2] and Goldblatt, Kahn, and Hanzal[4] have all pointed out that there is no progressive increase of arterial pressure after constriction of the aorta below the renal arteries.

In 4 of the 6 pregnant animals aortic compression was followed by a definite gradual rise of blood pressure; in the other 2 the rises were small (16 mm in 2 hours and 24 mm in one hour respectively). The rises were so gradual that one could not usually say exactly when they began. The rises under chloretone were clear-cut and amounted to 10-58 mm but no strictly hypertensive levels were reached, perhaps because of the very low initial values. In the animal under morphine and nembutal the blood pressure was more normal but the rise was small. The 2 decerebrate animals showed considerable (34 and 48 mm) rises of blood pressure, reaching in one case the definitely hypertensive level of 196 mm Hg.

* Supported by the John and Mary Markle Foundation.

1 Page, Ernest W., and Ogden, Eric, *Am. J. Obst. and Gyn.*, 1939, **38**, 230.

2 Brotchner, R. J., Proc. Soc. Exp. Biol. and Med., 1939, **40**, 264.

3 Rytand, D. A., *J. Clin. Invest.*, 1938, **17**, 391.

4 Goldblatt, H., Kahn, J. R., and Hanzal, R. F., *J. Exp. Med.*, 1939, **69**, 649.

TABLE I.
Blood Pressure Rise After Aortic Compression.

B.P. Mm Hg					
Before clamping	After clamping	Immediate rise	Subsequent rise	Duration of clamping (min)	
			Pregnant Animals		
148	196	20	28	42	Dog, decerebrate. Early pregnancy.
68	120	?	?	30?	
60	104	10	24	34	Cat, decerebrate. First observations after accidental occlusion of aorta. Late pregnancy.
86	104	8	10	225	
56	70	6	8	36	Dog, chloretone. Late pregnancy.
68	130	4	58	138	
64	76	4	8	87	Dog, morphine and nembutal. Late pregnancy. Very slight reductions in femoral pressure.
134	158	4	28	72	
164	110	8	—62	400	Dog, morphine and nembutal. Late pregnancy. First aortic compression extreme. Fetuses probably killed. Dog in bad condition at end of experiment.
54	80	10	16	36	
54	96	8	32	147	
62	98	10	26	130	Dog, chloretone. Late pregnancy
58	66	8	0	65	Same dog after removal of both uterine horns.
			Non-pregnant Animals.		
100	100	6	— 6	150	Dog, morphine and nembutal; non-pregnant female.
42	46	4	0	122	Dog, chloretone; male.
150	154	16	—12	26	Dog, nembutal; non-pregnant female.
144	148	14	—10	47	Dog, decerebrate; non-pregnant female.
98	116	20	— 2	93	

Figures in columns 1 and 2 measured immediately before compression and decompression respectively.
Column 3 represents highest point attained within 3 minutes of compression.
Figures in column 4 were obtained by subtraction to indicate the slow changes in blood pressure after the immediate adjustments had been made.

Release of the aortic clamp was followed by a return to the previous blood pressure, sometimes immediately, sometimes gradually during 20 minutes.

These rises of blood pressure could be produced repeatedly in the same animal except after a long period of unduly severe constriction, when it may be supposed that prolonged anoxemia had caused irreversible changes in the uterus or its contents.

From one dog, after several repetitions of the rise in blood pressure, the uterus and its contents were removed after clamping the mesosalpinx. It was then no longer possible to reproduce the slow rise of blood pressure by tightening the aortic clamp.

Since our 4 control animals exactly confirm Brotchner's finding that compression of the aorta below the renal artery produces no such prolonged rise as we have here described, we are forced to conclude that the products of conception (*i. e.*, fetus, placenta, or gravid uterus) are fundamentally responsible for these slow blood pressure rises. This conclusion is confirmed by the animal in which the blood pressure failed to respond to aortic constriction after the uterus and its contents had been removed. The fact that these characteristic rises in pressure began to appear and disappear only with tightening and loosening of the aortic clamp makes it hard to escape the conclusion that compression of the aorta is the factor in these experiments which determined the changes. It is possible that some nerve excitation originating from the constriction or manipulation might produce this effect in the gravid and not in the non-gravid animal, but on the grounds already discussed it seems to us very much more likely that the immediate determining factor is limitation of blood pressure or blood flow to the gravid uterus.

The attempt to show this effect in chronic experiments has been so far unsatisfactory in both dogs and rabbits because if the blood supply is not reduced beyond a point which would endanger the fetus or cause abortion, there is so much anastomotic circulation established within 24 hours that the femoral pressure is nearly equal to the carotid. Further steps are being taken to effect a more permanent diminution of blood supply to the gravid uterus.

If these experiments signify that circulatory insufficiency to the gravid uterus may produce a rise in systemic blood pressure in abnormal pregnancy, it may well be asked whether a balance between the actual uterine blood supply and the effects of temporarily inadequate blood supply may not play a part in the regulation of the circulation during normal pregnancy.

11092

Effect of Temperature on Bacteriostatic Action of Sulfanilamide upon Members of the Enterococcus Group.

Erwin Neter.

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Until recently, the members of the enterococcus group were considered to be rather resistant to the bacteriostatic and bactericidal action of sulfanilamide, both *in vivo* and *vitro*. Thus, Helmholz and Osterberg[1] found that sulfanilamide when given by mouth produces a urine strongly bactericidal for microörganisms usually found in urinary infections with the exception of the *Streptococcus fecalis*. Bliss and Long[2] reported that hemolytic enterococci (Lancefield group D) were not affected by sulfanilamide in a concentration of 1:10,000 in beef infusion, neopeptone broth, even when the broths were inoculated with relatively small numbers of microörganisms. In contradistinction to fibrinolytic hemolytic streptococci, several strains of hemolytic enterococci were not or only slightly inhibited in 1% dextrose broth containing 0.8% sulfanilamide, when one loopful of a 16 to 18 hours broth culture was used for inoculation (Neter[3, 4]). Long and Bliss[5] reported that hemolytic enterococci, Lancefield Group D, are resistant to the bacteriostatic action of sulfanilamide in concentrations up to 800 mg %; above this concentration a slight retarding of growth was noted with a few strains.

Recent experiments, however, revealed that under certain conditions it may be possible to demonstrate a definite bacteriostatic action of sulfanilamide upon members of the enterococcus group. Bliss and Long[6] found that the growth of enterococci may be definitely delayed, provided that high concentrations of sulfanilamide and small inocula are used. A marked bacteriostatic action of sulfanilamide in concentrations from 800 mg % to 1000 mg % upon hemolytic enterococci could be demonstrated,[7] employing as culture medium, 1%

1 Helmholz, H. F., and Osterberg, A. S., *Proc. Staff Meetings of the Mayo Clinic*, 1937, **12**, 377.

2 Bliss, E. A., and Long, P. H., *New England J. Med.*, 1937, **217**, 18.

3 Neter, E., *J. Bact.*, 1938, **36**.

4 Neter, E., *J. Lab. Clin. Med.*, 1939, **24**, 650.

5 Long, P. H., and Bliss, E. A., *The Clinical and Experimental Use of Sulfanilamide, Sulfapyridine and Allied Compounds*, 1939, 102.

6 Bliss, E. A., and Long, P. H., *Abstracts, Third Internat. Congress for Microbiology*, New York, 1939, 251.

7 Neter, E., Proc. Soc. Exp. Biol. and Med., 1939, **42**, 668.

maltose phenol red broth containing from 6.5% to 7% sodium chloride. In this broth the growth of hemolytic enterococci is markedly delayed. This culture medium was chosen because of the possibility that the delay in growth of the microörganisms may overcome the lag-period in the action of sulfanilamide. Bacteriostasis was also obtained with broth containing only ¼% maltose. Further experiments[8] revealed that hemolytic enterococci in 1% sulfanilamide broth, that failed to show visible growth upon incubation in contradistinction to the control, lost their viability more rapidly than the microörganisms in the control broth. On the other hand, the death rate of very small inocula of hemolytic enterococci, that failed to show visible growth in both the sulfanilamide and control broths, was not materially affected by sulfanilamide. Similar results were obtained with non-hemolytic enterococci. White and Parker,[9] and White[10] recently reported that sulfanilamide is more effective toward hemolytic streptococci at 39°C and 40°C than at 37°C. Wengatz, Boak, and Carpenter[11] showed that 0.01% of sulfanilamide may shorten by 50% the thermal death time of gonococci at 41.5°C. In view of these observations it was decided to determine the influence of temperature on the bacteriostatic activity of sulfanilamide toward members of the enterococcus group.

The experiments were carried out as follows: As culture medium, ¼% maltose phenol red broth base (Difco), containing tryptose (1%), sodium chloride (0.5%), dipotassium phosphate (0.1%) and phenol red, was used. To one part of this culture medium, 1% of sulfanilamide was added, to the other ½% of sodium chloride. Sodium chloride was added to the control broth in order to compensate for increased osmotic pressure of the sulfanilamide broth. The culture media were autoclaved at 15 pounds pressure for 15 minutes. Several strains of hemolytic and non-hemolytic enterococci were used; 2 of these strains were obtained through the courtesy of Dr. J. M. Sherman, Ithaca, New York. Decreasing amounts (volume 0.1 cc) of the respective broth culture were used for inoculation of sulfanilamide and control broths (volume 5 cc). Parallel experiments were carried out at 37°C and 43°C, respectively. Visible growth was noted at various intervals.

Table I presents the results of an experiment in which the bacteriostatic action of 1% sulfanilamide toward a strain of non-hemolytic enterococcus was tested, both at 37°C and 43°C. It may

8 Neter, E., *J. Bact.*, 1940, in press.

9 White, H. J., and Parker, J. M., *J. Bact.*, 1938, **36**, 481.

10 White, H. J., *J. Bact.*, 1939, **38**, 549.

11 Wengatz, H. F., Boak, R. A., and Carpenter, C. M., *J. Bact.*, 1938, **35**, 36.

TABLE I.

Bacteriostatic Action of Sulfanilamide (1%) on *Streptococcus fecalis* in ¼% Maltose Phenol Red Broth at Different Temperatures.
Inoculum from 48-hour Culture (0.1 cc).

		Dilutions.									
		I 1/50		II 1/2500		III 1/125,000		IV 1/6,250,000		V 1/312,500,000	
	Hr of incubation	C	S	C	S	C	S	C	S	C	S
		Incubation at 37°C.									
1.	16	+		+	—	—	—	—	—	—	—
2.	24	+	+	+	—	+	—	+	—	—	—
3.	48	+	+	+	+	+	—	+	—	—	—
4.	72	+	+	+	+	+	—	+	+	—	—
5.	96	+	+	+	+	+	—	+	+	—	—
6.	120	+	+	+	+	+	+	+	+	—	—
		Incubation at 43°C									
1.	16	+	—	+	—	—	—	—	—	—	—
2.	24	+	—	+	—	+	—	+	—	—	—
3.	48	+	—	+	—	+	—	+	—	—	—
4.	72	+	—	+	+	+	—	+	+	—	—
5.	96	+	—	+	+	+	—	+	+	—	—
6.	120	+	—	+	+	+	—	+	+	—	—

C = 1% NaCl ¼% Maltose Phenol Red Broth.
S = 1% Sulf. ½% NaCl ¼% Maltose Phenol Red Broth.

— = No visible growth.
+ to ++++ = Various degrees of visible growth.

be seen from this table that (1) sulfanilamide at 37°C delayed the growth, but did not completely prevent it, even when small inocula (1/6,250,000 dilution of a 48 hours culture) were used; (2) sulfanilamide at 43°C completely and continuously inhibited visible growth of the enterococcus; (3) sulfanilamide at 43°C was bacteriostatic toward much larger numbers (1:50 dilution of a 48 hours culture) of enterococci than at 37°C.

This increased bacteriostatic activity of sulfanilamide in concentration of 1% at 43°C could be demonstrated with 3 strains of hemolytic and 3 strains of non-hemolytic enterococci, even when relatively large numbers of microörganisms (1:50 dilution of a 18 to 48 hours culture) were used for inoculation. With one strain of non-hemolytic enterococcus, growth was not completely inhibited but definitely retarded by sulfanilamide at 43°C.

It is important to mention that the growth of the enterococci in the control broths was not markedly delayed or suppressed at 43°C in comparison to that obtained at 37°C. This observation supports the view of White,[10] namely, that the increase in activity of sulfanilamide toward hemolytic streptococci cannot be explained solely on the basis of a deceleration of the growth rate at higher temperature.

In conclusion, at 43°C sulfanilamide in concentration of 1% is markedly more bacteriostatic toward both hemolytic and non-hemolytic enterococci, than at 37°C.

11093

Tyrosinase in Feather Germs.

DONALD R. CHARLES AND MARY E. RAWLES. (Introduced by B. H. Willier.)

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The presumable enzyme system involved in melanin formation by growing feathers does not seem to have been investigated, although it might be supposed to be a tyrosinase or dopa-oxidase, by analogy with melanogenesis in mammalian skin and hair roots, amphibian skin, insect hypoderm, etc. That a tyrosinase *is* present, whatever its rôle, in feather germs of black and red chickens, and absent or somehow masked in the germs of certain white breeds, is shown by the following study.

Feather germs, in which the rhachis tip had not yet (or just re-

cently) broken through the sheath, were plucked from New Hampshire Red, Black Minorca, hybrid black (from Barred Plymouth Rock ♀ x R.I. Red ♂) or White Leghorn chickens. At this stage the germs were about 2-3, 1.5-2 and 1-1.5 cm long in reds, blacks and whites respectively and weighed about 75-100, 40-50 and 35 mg each. The feather sheath was split longitudinally, the papilla (which forms most of the mass of the germ) removed, and the barbs scraped off the inner surface of the sheath. About 150-750 mg of live barb tissue were thus obtained from 25-50 feather germs of one color type. They were immersed in 2 cc of M/120 pH 7.4 phosphate buffer and ground with a small amount of sand. In some cases the resulting mixture was centrifuged for 15 minutes at about 2000 r.p.m. and the supernatant fluid (approximately 1.5 cc) divided equally among 2 to 4 serological tubes. In other tests the mixture was divided and the portions centrifuged separately so that subsequent reactions occurred in the presence of precipitated solid barb material. Reagents were added to each tube in such concentrations and quantities as to bring the reaction mixtures to the compositions shown in Table I. Where urethane was not used a small crystal of thymol was added. The tubes were plugged with cotton and set in an incubator at 37°C.

TABLE I.
Tests for Tyrosinase in Colored Feather Germs and for Tyrosinase Inhibitor in Dominant White Germs.

	Composition of test mixture*				Melanin formation		
					Positive trials	Doubtful trials	Negative trials
1.	black†				0	0	3
2.	”		tyrosine		7	2	2
3.	”		”	urethane	8	0	0
4.	”		”	KCN	0	0	9
5.	”	boiled	”		0	0	4
6.	”		”	white	1	1	4
7.	”		”	” boiled	2	0	3
8.	red				0	0	2
9.	”		tyrosine		4	1	2
10.	”		”	urethane	3	0	2
11.	”		”	KCN	0	1	3
12.	”	boiled	”		0	1	1
13.	”		”	white	2	2	1
14.	”		”	” boiled	3	1	1
15.	white		tyrosine		0	0	4

* Volume of each mixture, about 1 cc. Concentrations in mixtures: tyrosine, 0.1-0.2%, including undissolved crystals; phosphate buffer pH 7.4, M/40; cyanide, M/500; urethane, 5%. Each portion of extract, from about 50-750 mg of live tissue, *i. e.*, juicy barb portions of 10-50 feather germs.

† Lines 1-7 are the combined data from Black Minorca and hybrid black barbs.

The freshly-prepared extracts were ordinarily very clear, slightly grayish if from black, pale orange from red, pinkish from white germs. They remained so, or became whitish and somewhat opaque, where negative reactions are recorded in Table I. In trials recorded as positive, whether from black or red germs, a disc of dense brown-black or black appeared at the top of the liquid in 6-30 hours and spread slowly downward. That this pigment was really melanin was not tested although it seemed very probable from the composition of the mixtures.

The reactions were not very constant among comparable mixtures. Of 30 tubes containing colored germ extract and tyrosine, with or without urethane (Table I, lines 2, 3, 9, 10), 22 showed definite melanin formation, 6 were negative and 3 doubtful. The same lack of reproducibility has been found in mammalian skin extracts.[1, 2]

In spite of the defects of the method, one conclusion can scarcely be escaped: birds, or at least chickens, like rabbits,[1] mice,[2] and amphibian larvae[3] do not lack tyrosinase. Five extracts of colored barbs (lines 1 and 8) without added tyrosine did not form melanin; but 22 of 31 extracts with tyrosine did. Tyrosine alone at pH 7.4 is well known to be relatively stable; and 5 of 6 mixtures of tyrosine with boiled extract (lines 5, 12) did not react; the sixth was dubious. Together these results show only that colored extracts contain a heat-labile substance necessary for melanin formation from tyrosine. That the substance is an enzyme of the oxidase class is suggested by the cyanide effect on its action (lines 4 and 11): 12 of 13 tyrosine-extract-cyanide mixtures failed to form melanin and one gave a doubtful reaction. The behavior of the extracts thus fits the classical definition of tyrosinase: an oxidase system in the presence of which tyrosine forms melanin, without added peroxide.

Several other more uncertain conclusions are suggested by the data of Table I. (1) Red feather germs seem to contain a tyrosinase like, if not identical with, that of black germs, producing *black* pigment *in vitro* as do red guinea pig hair follicles in dopa solution.[4] (New Hampshire Reds have some black or red and black feathers but the germs used were from regions of red plumage.) (2) White Leghorn germs contain little or no tyrosinase, or else have also a tyrosinase inhibitor as Hadley[5] and others seem to have supposed, on genetic grounds. (3) Such a substance, if it exists, is present in too small

1 Pugh, C. E. M., *Biochem. J.*, 1933, **27**, 475.

2 Charles, D. R., *Genetics*, 1938, **23**, 523.

3 Figge, F. H. J., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 569.

4 Russell, W. L., *Genetics*, 1939, 645.

5 Hadley, P. H., *Agric. Exp. Station R. I. State Coll. Bull.* 11, 1913, **155**, 151.

amounts to give a clear tyrosinase inhibition when extracts of roughly equal amounts of black and white tissue are mixed (lines 6, 7, 13, 14). (4) Five percent ethyl urethane seems to accelerate and perhaps intensify the tyrosinase action of feather germ extracts: positive reactions in the presence of urethane appeared at 5-18 hours; without urethane, at 20-40 hours. This effect may come about by blocking out certain reducing systems involving dehydrogenases.

The variability of results is perhaps explained by the morphological aspect of pigment formation in feather germs. The melanophores in which the melanin seems to be produced are only a small part of the barb tissue. They are fully active, possibly, only in a narrow circumferential band at the base of the germ. If these cells alone contain an appreciable amount of tyrosinase, extract of 100 mg of barb would represent only a few mg of tyrosinase-containing tissue. It might be expected that much or all of the enzyme would sometimes be destroyed during extraction.

Summary. Extracts of black chicken feather germs show a cyanide-sensitive, heat-labile tyrosinase activity, forming melanin from tyrosine without added peroxide, in about 80% of trials when one cc of the reaction mixture contains extract of 50-750 mg of young barb tissue. Extracts of red feather germs show a similar activity in about 60% of cases, forming black rather than red pigment. Extracts of White Leghorn germs do not inhibit the tyrosinase from roughly equivalent amounts of black germ.

11094 P

Inhibition of Experimental Dental Caries by Fluorine in the Absence of Saliva.*

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Although the inhibition of dental caries by fluorine has been demonstrated in endemic areas[1, 2] and in laboratory rats fed on a

* The investigation upon which this report is based was made possible by funds granted by the Carnegie Corporation of New York.

† The author wishes to thank Drs. Harold C. Hodge and Sidney B. Finn for their kind cooperation in this experiment.

1 Black, G. V., and McKay, F. S., *D. Cosmos*, 1916, **58**, 129.

2 Dean, H. T., *Pub. Health Rep.*, 1938, **53**, 1443.

caries-producing diet[3, 4] the mechanism by which the action takes place has not yet been adequately demonstrated. Armstrong and Brekhus[5] found a higher fluorine content of sound enamel than in carious and offered the hypothesis that this may be the factor responsible for the inhibition. In this report we have attempted a further investigation of this mechanism. Since the enamel of the molar teeth of the animals used was fully formed before fluorine was applied it would appear that the action of fluorine must be due to its being held in the region of the tooth surface or, as far as the dentin is concerned, to the possibility of fluorine reaching it through the circulation.

Previous studies are inclined to disregard the ability of fluorine becoming incorporated into the tooth surface after formation and thus causing an increased resistance to the mechanism responsible for decalcification because the rôle exerted by saliva could not be accurately determined. By using rats in which all the principal salivary glands had been removed[6] we have practically eliminated the possibility of fluorine acting through the saliva. Further, since the fluorine was administered as potassium fluoride in a drop of water in our experiments it is not likely that the fluorine would remain in the mouth in this form sufficiently long to have a prolonged effect. However, evidence is presented that fluorine administered in this way can decrease the incidence of caries in spite of the interference with the salivary secretions or mode of administration.

Experiments and Results. A total of 76 rats were selected from the same Wistar breeding stock at weaning. At 22 days of age the salivary glands were removed from 32 of the animals. On the 31st day all animals were placed upon a caries-producing diet[7] and an aqueous dosage of 3 mg of fluorine was administered to 12 of the operated animals. The food and distilled water were given *ad lib.* Weights were taken every 4 days. After 200 days on the diet, the animals were chloroformed, the jaws separated, and all molar teeth examined by means of a fine-pointed explorer under a binocular microscope, magnification 30×. An attempt was made to classify lesions into simple fractures and carious cavities. Male and female differences were not sufficiently significant to warrant independent consideration.

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3 Miller, B. F., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 389.

4 Hodge, H. C., and Finn, S. B., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 318.

5 Armstrong, W. D., and Brekhus, P. J., *J. D. Res.*, 1938, **17**, 393.

6 Cheyne, V. D., *J. D. Res.*, 1939, **18**, 457.

7 Hoppert, C. A., Webber, P. A., and Canniff, T. L., *J. D. Res.*, 1932, **12**, 161.

TABLE I.

Group	Condition of animal	No. of animals	No. of molar teeth examined	No. fractured cusps per animal	No. carious cuspal involvements per animal	No. carious teeth per animal	No. whole teeth destroyed per animal
I	Normal	44	528	1.6	9.1	3.5	0.8
II	Extirpated salivary glands	20	240	0.2	40.0	10.5	6.7
III	Extirpated salivary glands (plus 3 mg fluorine per day)	12	144	4.3	6.0	2.3	0.3

As may be noted from Table I, the reduction of saliva greatly increases the incidence of rat caries. This is in accord with a previous demonstration by us.[8] Normal animals on the caries-producing diet average 9.1 cuspal involvements and 3.5 carious teeth per animal; those with salivary glands extirpated average 40.0 cuspal involvements and 10.5 carious teeth per animal; and desalivated rats receiving 3 mg of fluorine per day average 6.0 cuspal involvements and 2.3 carious teeth per animal. In all the rats the lower teeth were more involved than the upper. Since the average number of carious teeth in the last group is lower than normal it seems that the beneficial effect of fluorine is more potent than that of the saliva acting independently. Likewise, a reduction of 80 to 85% of these carious afflictions in these desalivated rats receiving fluorine (Group III, Table I), shows that the fluorine has a caries-inhibiting effect even when oral secretions are at a minimum. The relative frequency of occurrence of fractures and advanced cavities in the control and fluorine groups suggests that while the fluorine does not prevent the initial fracture of the teeth it does prevent the subsequent progressive destruction. These findings indicate that the fluorine has a direct and persistent action on the tooth or in the region where food and bacteria come in contact with the tooth surface and in this way retards the progress of decay.

11095 P

Degeneration of the Infundibular Nerve Fibers Without Precipitating Diabetes Insipidus.*†

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In a previous report[1] we demonstrated that the denervation theory can not hold rigidly in explaining the causation of d. i. providing the elaboration of the antidiuretic principle is confined solely to hypophysial tissue. Our experiment does not invalidate the denervation theory if hypothalamic tissue possess antidiuretic secretory powers.

8 Cheyne, V. D., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 587.

* Aided by a grant from the Rockefeller Foundation.

† Pars nervosa is used as synonymous to Wislocki's[7] usage of neurohypophysis.

1 Keller, A. D., and Hamilton, J. W., *Arch. Surg.*, 1938, **37**, 760.

Sato[2] and Trendelenburg[3] suggested that the hypothalamus does possess latent antidiuretic secretory powers. This appeared less remote after Geiling's[4] strong evidence pointing to the pars nervosa as elaborating the antidiuretic principle. Scharrer[5] has postulated a secretory function for the cells of the supra-optic and paraventricular nuclei and Finley's[6] work could well support such an interpretation. Wislocki[7] and particularly Gersh,[8] on the basis of indirect anatomical evidence, would limit the antidiuretic secretory powers solely to the pars nervosa.

With a view of attempting to gain further insight into the aforementioned problem the following experiment was executed in 8 cats. The ventral portion of the hypothalamus, particularly that portion adjacent and posterior to the optic chiasm, was first isolated from the rest of the hypothalamic tissue and then removed with forceps. This insured complete separation of the hypophysis from the hypothalamus with more encroachment upon the hypothalamus than in our previous experiment[1] and also eliminated the possibility of leaving infundibular nerve fibers taking origin from cells located in the hypothalamic tissue immediately adjacent to the hypophysial stalk.

The cats were maintained on a daily food intake of 100 cc undiluted evaporated milk and 50 g of Baloration. Water was available for 22 hours of each 24. Five of the cats were sacrificed from 2 to 8 months after operation. the remaining 3 are still under observation. The water exchange showed no deviation from the normal in 7 of the 8 cats, while one exhibited a mild increase. The water exchange exhibited no noteworthy increase during thyroid and salt feeding.

Histological studies showed that infringement upon the tissue of the pars nervosa (central sloughing with enlargement of the infundibular cavity) was greater than in our former experiments.[1] In instances there was essentially no tissue of the pars nervosa remaining, certainly there was little likelihood that the fragments which remained contained functional secretory cells. In instances there was no drastic disappearance of the cells of the supra-optic nuclei. This was appreciated in our original series but was not reported, except privately, because the question was raised as to whether our termination dates allowed sufficient time for retrograde degeneration to occur.

2 Sato, G., *Arch. Exp. Path. Pharm.*, 1928, **131**, 45.

3 Trendelenburg, P., *Klin. Wschr.*, 1928, **131**, 45.

4 Geiling, E. M. K., *Bull. Johns Hopkins Hosp.*, 1935, **57**, 123.

5 Scharrer, E., *Z. f. d. ges. neurol. n. psychiat.*, 1933, **145**, 462.

6 Finley, K. H., *A. R. N. M. D.*, 1938, **18**, 94.

7 Wislocki, G. B., and King, L. S., *Am. J. Anat.*, 1936, **58**, 421.

8 Gersh, I., *Am. J. Anat.*, 1939, **64**, 407.

These cells frequently degenerate following operative procedures in the region of the hypophysial stalk. Indeed it is difficult to section the stalk through its proximal extent without degeneration of these nuclei. The fact that these cells do not always degenerate following section of the infundibular nerve fibers does not question that their axis cylinders contribute to the "infundibular nerve trunk." It does demonstrate that factors other than mere section of the axis cylinders are responsible for their disappearance.‡

In view of Sato's and Scharrer's work could it be possible that the cells of the supra-optic nuclei, or other hypothalamic tissue, elaborate sufficient antidiuretic substance to maintain a normal fluid exchange in the absence of the tissue of the pars nervosa and ventral hypothalamus? We have found that separating the hypophysis and the hypothalamus at the junction of the two frequently results in d. i. in the cat and usually in the dog. It is true that these experiments, as well as in the series of partial and total hypophysectomies,[9] there has been a rough correlation between the appearance of d. i. and the degeneration of the supra-optic nuclei. Whether or not this association is merely coincidental is as yet problematic.

Conclusions and Comments. The tissue of the pars nervosa in its entirety plus that of the ventral extent of the hypothalamus is not essential for the maintenance of a normal fluid exchange in the cat. Therefore, unless a tissue of the adenohypophysis secretes the antidiuretic principle, tissue elsewhere (hypothalamus) must possess this power. This conclusion is predicated on the assumptions, first, that these experiments were not complicated by as yet unrecognized factors, and, second, that a continuous elaboration of the antidiuretic substance is invariably essential for the maintenance of a normal fluid exchange.

If the adenohypophysis is concerned the pars tuberalis as well as the pars intermedium possess antidiuretic secretory powers since: (1) total hypophysectomy except for remnant of tuberalis,[9] and (2) removal of the entire tuberalis and most of the pars anterior, leaving the isolated posterior lobe, can be accomplished without precipitating d. i.

‡ Such factors might be (1) devitalization of cells distal to the tissue defect when electrolytic lesions are made, (2) pulling upon the cell bodies by tension exerted through the axis cylinders during stalk sectioning procedures, and (3) vascular disturbances sufficient to cause death of the nerve cells without regional gliosis and demyelinization.

9 (a) Keller, A. D., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 31; (b) Keller, A. D., *Am. J. Physiol.*, 1938, **123**, 116; (c) Keller, A. D., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 555.

11096 P

Effect of Metrazol on Recent Learning.

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One of the theories attempting to explain the mechanism of metrazol treatment in the psychoses is that a noxious or destructive effect is exerted on the central nervous system so that the mental symptoms, affecting neural processes of the highest order (largely cortical), and also of most recent origin, are the most vulnerable and hence the first to disappear. In order to further test the validity of this concept we attempted to establish a neural pattern of recent origin and see if it could be abolished by the administration of metrazol.

The demonstration required the establishment of new learnings of a fairly high order which were amenable to objective experimental control, observation and measurement. The code transcription procedure was chosen as a simple technic which would satisfy these criteria.

The following routine was observed for the experiments: After preliminary explanations and demonstration, the patient was asked to transcribe from memory the nonsense syllable code in the appropriate blank spaces as rapidly as possible. He worked 4 minutes; his score was the number of items transcribed. After 2 minutes' rest he was given 6 minutes of practice at transcription. Then followed another 2-minute rest interval and a 4-minute retest. The number of items transcribed in this latter period constitute the "Retest 1" score. During the week following Retest 1, two or 3 metrazol injections were administered. At the end of the week another 4-minute retest was given; the number of items transcribed constitute the results of Retest 2. By comparing the scores made on Retest 1 and 2, any change in the patient's performance could be determined.

An alternate and comparable form of the code transcription test was used on the same patients (Control I). In these control experiments the patients were subjected to the same procedure, but during the period of forgetting no metrazol injections were given. The tests were also given to a control group not receiving metrazol. (Control II).

This report is based on 6 patients and 6 controls. All were young or middle-aged adults. The cases included 2 of chronic encephalitis; one of depression of the manic depressive psychosis;

one involutional melancholia and 2 schizophrenics. Included in the controls were 4 normals, one chronic encephalitis and one recovered hypo-manic (manic depressive psychosis). Every effort was made to keep the experimental conditions constant. Patients with disturbing mental symptoms were not studied, the subjects used being selected on the basis of their ability to coöperate. The factor of attention was more or less uniform throughout the test periods as far as could be ascertained. The test results for 3 patients who received control tests both before and after treatment showed no significant difference from the others of the group.

Since the individuals responded in a fairly uniform manner a short tabular summary of the average results in each group is submitted:

Average difference in scores of Retests 1 and 2:

Metrazol cases	—25.1%	27.8% difference
Control I	+ 2.7%	
Control II	+ 3.5%	28.6% "

The data show that performance of the code transcription after metrazol injection resulted in a score of 27.8% lower than control tests on these same patients. The control subjects (Control II), those not receiving metrazol, showed no mean loss at all, but a small mean gain of 3.5% after the lapse of the time interval.

Although the series is small, the results are statistically reliable. The impairment in learning with metrazol would appear to be due to impaired memory. A number of factors entered into the learning procedure. The question of impairment of attention as against impairment of memory is being given further study.

11097

Isolation of Antibody from Agglutinate of Type I Pneumococcus by Treatment with Acid.

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In previous reports from this laboratory[1, 2] it has been shown that the liberation of antibody from immune precipitate of Type I Pneu-

1 Liu, S. C., and Wu, H., *Chinese J. Physiol.*, 1938, **13**, 449.

2 Liu, S. C., and Wu, H., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 144.

mococcus by the action of dilute acid or alkali is due to a shift of the antigen-antibody equilibrium as follows:

$$B_mG_n \underset{\text{neutral}}{\overset{\text{alkali or acid}}{\rightleftharpoons}} B_{m-x}G_n + xB$$

Where G = antigen, B = antibody, and B_mG_n = immune precipitate formed in neutral solution. The amount of antibody set free (xB) increases with acidity or alkalinity, but so also is the solubility of $B_{m-x}G_n$ which would decrease the yield by recombining with B when the solution is neutralized. Not all the antibody liberated by acid or alkali is thus recoverable, and there is in either acid or alkaline solution an optimum pH for the recovery.

The liberation of antibody from the agglutinate by the action of acid or alkali is probably due to a similar shift of equilibrium, but there is an important difference between the immune precipitate and the agglutinate. Whereas the immune precipitate and its partial dissociation product ($B_{m-x}G_n$) are more or less soluble in acid or alkaline solution, the agglutinate or its dissociation product is not soluble as long as the cells are intact. It can, therefore, be completely removed by centrifuging and all the antibody set free should be recoverable. The purpose of the present study is to test this point.

Preliminary experiments showed that Type I Pneumococcus vaccine treated with alkali (even at pH 8), gave off an appreciable amount of nitrogen, while treatment with acid (pH 5 to 1.8) caused practically no loss of nitrogen. Hence in the present study the agglutinates were treated only with acid.

Effect of pH on the dissociation of Type I Pneumococcus agglutinate. In a series of 15-cc centrifuge tubes 4 cc of immune rabbit serum, and 6 cc of washed, heat killed Type I Pneumococcus vaccine were mixed. The mixtures were incubated at 37°C for 2 hours, and then kept in ice box over night and centrifuged in the cold. The agglutinates were washed 3 times with 4 cc portions of ice cold normal saline and then suspended in 6 cc of distilled water. Equal volumes of HCl of different concentrations were added with constant stirring. After 10 minutes the mixtures were centrifuged at high speed.* The centrifuge tubes were tightly covered with tin foil and rubber band to prevent loss of water by evaporation. Water clear supernatants were obtained. The nitrogen contents of the supernatants and residues were determined and the pH's of the supernatants were measured with a glass electrode. To aliquot portions of the supernatants were added 10% NaCl solution to bring

* We used a Multispeed Attachment on Size 2 centrifuge made by International Equipment Co., Boston.

TABLE I.
Effect of pH on Liberation and Recovery of Antibody from Agglutinate of Type I Pneumococcus.

pH	A Nitrogen in vaccine, mg	B N in residue after acid treatment, mg	C N in acid supernatant, mg	D = B + C Agglutinate N, mg	E = D − A Agglutinin N, mg	F N in neutral supernatant, mg	$\frac{F}{E} \times 100$ Recovery of antibody, %
2.03	6.59	6.49	8.65	15.14	8.55	7.45	87.1
2.30	"	6.71	8.35	15.06	8.47	7.30	86.2
2.68	"	7.37	7.58	14.95	8.36	6.75	80.6
3.03	"	7.85	6.80	14.65	8.06	6.10	75.7
3.35	"	8.84	6.12	14.96	8.37	5.64	67.2
4.08	"	10.68	4.35	15.03	8.44	4.10	48.6

the concentration of NaCl to 0.9%, and then neutralized with NaOH. After standing for 20 minutes the mixtures were centrifuged. The supernatants (antibody) and the precipitates (probably acid denatured antibody) were analyzed for nitrogen. The results are shown in Table I.

It has been found in another experiment that treatment of washed vaccine with normal rabbit serum and then with HCl causes a loss of N amounting to about 12% of the total nitrogen in the vaccine. This interesting phenomenon is being studied further. In Table I the figures for vaccine N have been corrected for this loss.

From Table I it is seen that the dissociation of agglutinate of Type I Pneumococcus increases with acidity. At pH 2 the dissociation is complete, as the amount of N in the supernatant is, within the limits of error, equal to the amount of N in the agglutinin. The amount of antibody recovered after neutralization, however, is only about 87%, due probably to some denaturation.

Repeated use of the vaccine obtained from dissociated agglutinate. Thirty cc vaccine containing 16.7 mg N were mixed with 15 cc immune rabbit serum. The agglutinate was washed 3 times with cold saline and then treated with 25 cc of N/30 HCl and centrifuged. The vaccine residue left after removal of agglutinin was washed twice with N/30 HCl, suspended in 15 cc normal saline and neutralized with NaOH. This neutralized vaccine was again used for preparation of agglutinate with 15 cc immune serum. This procedure was repeated 3 more times. Microscopic examination showed that the cells were still Gram positive diplococci. The purity of the recovered antibody was determined by the method of Heidelberger and Kabat.[3] The results are shown in Table II.

It is seen that the vaccine, after repeated use, shows no decrease of antibody-combining power as shown by the amount of N in the acid supernatant. The recovery of antibody seems to show a slight improvement with repeated use of the vaccine, but this point requires further study. The degree of purity of the antibody recovered in each of the 4 successive agglutinations is at least 95%.

Summary. By treating the agglutinate of Type I Pneumococcus with dilute HCl (pH 2), all the antibody is liberated, and after removing the cells and neutralizing, as much as 87% may be recovered. The recovered antibody is at least 95% pure as shown by specific precipitation followed by agglutination. The vaccine can be used repeatedly for the isolation of the antibody. The method thus combines simplicity of procedure with good yield and high purity of product.

[3] Heidelberger, M., and Kabat, E. A., *J. Exp. Med.*, 1938, **67**, 181.

TABLE II.
Recovery of Antibody from Agglutinate Prepared from Repeatedly Used Vaccine.

No. of times the vaccine was used	Recovery of antibody			Purity of antibody				
	N in acid supernatant, mg	N in neutralized supernatant, mg	Recovery, %	Total N in antibody solution used, mg	Precipitin N, mg	Agglutinin N, mg	Total antibody N, mg	Antibody N / Total N, %
1*	22.75	17.38	76.4	.638	.522	.080	.602	94.3
				.319	—	.288	.288	90.2
2	24.50	19.47	79.5	.708	.575	.107	.682	96.3
				.354	—	.316	.316	89.3
3	25.40	21.01	82.7	.708	.581	.096	.677	95.6
				.354	—	.316	.316	89.3
4	25.40	21.93	86.4	.568	.490	.055	.545	95.9
				.284	—	.255	.255	89.7

*The vaccine contained 16.7 mg of nitrogen.

11098

Rapid Multiplication of *Mycobacterium tuberculosis* in Chinese Hamsters.

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The laboratory diagnosis of tuberculosis by the usual guinea pig inoculation method is admittedly sensitive and reliable in experienced hands and at times almost indispensable. On the other hand, the method contains several defects, one of which is the length of time generally required to arrive at the desired information. If a method is available which could decrease the time factor without interfering with the sensitivity of the susceptible animal tissue it would appear to be ideal for diagnosis. Negre and Bretey[1] found that the accuracy of guinea pig inoculation could be enhanced for diagnostic purpose if the animals were subjected first to a prolonged series of injection with an acetone extract of acid-fast bacilli. Such a procedure, while admittedly useful under special conditions, does not appear to decrease the time factor. Pickof[2] reported that by injecting the suspected specimen into an area of the guinea pig skin previously filled with a suspension of Silica powder diagnosis of acid-fast bacilli could be made within 10 to 12 days. It appears that such a procedure may well be applied to the Chinese hamster, an animal which has been found to be equally if not more susceptible to the tubercle bacilli than the guinea pig.[3–6] Apart from this the initial low cost, the small cost of maintenance and the abundance of this animal in this part of the country have much to recommend their use. In the present communication we have attempted to compare the usefulness of Chinese hamsters to that of the guinea pig under experimental conditions for the rapid diagnosis of *Mycobacterium tuberculosis* and for the differentiation between human strains and possibly other acid-fast bacilli is described.

The technic described by Pickof[2] was closely followed with the exception that silica powder was replaced by kaolin (unpurified). In

1 Negre, L., and Bretey, J., *Am. Rev. Tuberc.*, 1938, **38**, 531.
2 Pickof, F. L., *Am. J. Clin. Path.*, 1939, **9**, 339.
3 Korns, J. H., and Lu, Y. C., *China Med. J.*, 1927, **41**, 234.
4 Korns, J. H., and Lu, Y. C., PROC. SOC. EXP. BIOL. AND MED., 1927, **24**, 807.
5 Korns, J. H., and Lu, Y. C., *Am. Rev. Tuberc.*, 1928, **17**, 279.
6 Lu, Y. C., *Ibid.*, 1929, **20**, 938.

the first experiment it was considered of interest to compare the relative susceptibility of the 2 species of animals to tuberculosis. For this purpose 11 guinea pigs weighing about 300 g each and 15 hamsters were inoculated with a virulent human strain of *Mycobacterium tuberculosis*. The suspension of the organism was made by prolonged grinding of an accurately weighed amount of moist culture grown on Lowenstein medium for 4 weeks. Then it was suspended in saline in proportion of 1 per 1000 of saline. Serial dilutions were made from this and the dilution showing 1 bacillus per 10 fields when viewed under high magnification (970 ×) was used. The inoculum consisted of 0.1 cc of the suspension for both kinds of animals, inoculation being made on each animal, one on each lateral aspect of the trunk. It is of interest to note that inoculations could be easily made into the delicate loose skin of the hamsters. Examination of all animals were made at the end of 2 weeks. In order to obtain as much material as possible for examination animals were anesthetized, the skin was incised and the caseous material formed at the site of inoculation was smeared on glass slides. Employing such a procedure it was found that the agreement between the 2 species of animals was complete and that the acid-fast bacilli could be demonstrated in practically all the specimens although there were marked variations in the number of organisms from both species of animals. This indicates that the number of animals employed and the number of sites injected are important factors upon which a successful diagnosis of any given specimen depends. As far as this experiment goes the results indicate that Chinese hamsters are just as useful as guinea pigs for the diagnosis of tuberculosis by the Pickof technic.

Since hamsters have been demonstrated to be suitable, it was considered of interest to determine the earliest time at which positive results may be obtained. The size of the inoculum and the bacterial suspension were the same as those used above. Fifteen hamsters were used. Examinations were made on the 5th, 8th, and 11th day, 5 animals being examined each time. It was found that positive findings were obtained on the 8th day in about 50% of the specimens examined although the number of acid-fast bacilli found was significantly less than those observed in the first experiment in 2 weeks. On the 11th day the specimens from all the animals were positive for acid-fast bacilli. The results of this experiment complement those obtained in the first experiment insofar as the usefulness of hamsters for the rapid diagnosis of tuberculosis is concerned.

In the third experiment it was considered of interest to determine whether the method is applicable to other acid-fast bacilli such as

Mycob. bovis, Ravenal strain; *Mycob. tuberculosis* strain H37A, BCG, *Mycob. avium* strain 531, and *Mycob. smegmatis.* The above organisms were obtained from the Phipps Institute and represent virulent, attenuated, and saprophytic strains from various animal sources. Ten hamsters were used for each organism. Three- to 4-week-old cultures grown on appropriate solid medium of all the cultures except *Mycob. smegmatis* were used. In the case of the latter 10-day-old culture was employed. Subcultures from the original slant were made to ensure viability. Suspension and standardization of the inoculum for each organism were made in the same manner as experiment I with the exception that the dilution containing approximately 1 bacillus per every other field was used. The amount of inoculum and the technic of inoculation were essentially the same as those employed in the previous experiments. Examinations made at the end of 2 weeks revealed 2 distinct differences. On the one hand it was found that no acid-fast bacilli could be demonstrated in any of the specimens obtained from animals inoculated with BCG, *Mycob. avium* and *Mycob. smegmatis.* On the other it was found that organisms could be demonstrated in the specimen obtained from animals inoculated with *Mycob. bovis* and *Mycob. tuberculosis* H37A. In case of the former organism acid-fast bacilli were easily found in all the specimens while demonstration of the latter was more difficult. This difference in the ability of proliferation of the 2 organisms in the animal tissue may in some measure be accounted for by the greater virulence of the bovine organism. The results indicate that under the conditions of the experiment, a differential diagnosis can be made in hamsters insofar as the difference between human and bovine strains on the one hand and the avian and non-pathogenic acid-fast bacilli on the other is concerned. The application of this method to clinical materials is being studied.

Conclusion. Chinese hamsters are suitable for the rapid diagnosis of tuberculosis. Positive findings may be obtained in from 10-14 days. Non-pathogenic acid-fast bacilli gave negative results.

11099

Comparative Activity of Vitamin H Curative of Egg-White Injury Administered Orally and Parenterally to Rats.

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The factor (vitamin H) curative of egg-white injury in rats is insoluble in water in its natural state as it occurs in liver, kidney or yeast. It can be extracted from yeast, however, by autolysis with water in the presence of toluene and from liver by proteolytic digestion or by hydrolysis at high pressure.[1–4] The curative action of vitamin H can be demonstrated by yeast, liver and kidney as such only in oral administration, because these substances cannot be given in any other manner. Their activity when they are parenterally administered,[2, 3] however, can be shown by injection of concentrates in aqueous solution.

For the quantitative study of different concentrates, it became important to establish the comparative strength of vitamin H when it is administered orally and parenterally. The growth-promoting activity of riboflavin (lactoflavin) is independent of the way in which it is given, that is, whether orally or parenterally.[5] On the other hand, it is a well-known clinical fact that the factor in liver curative of pernicious anemia is much more active in parenteral than in oral administration.

After the parenteral route proved to be more efficacious in preliminary bioassays of vitamin H concentrates, quantitative data were secured from experiments in which the preparation was tested simultaneously on two groups of rats; to one group it was administered orally, to the other parenterally. The samples tested were of different degrees of purification and had been prepared from yeast or liver. The dry residue contained in 1 oral rat day dose (unit) varied between 70 micrograms in sample K-554 and 150 mg in sample K-5. Rats in which the egg-white injury remained unin-

1 Lease, J. G., and Parsons, H. T., *Biochem. J.*, 1934, **28**, 2109.

2 György, P., in Pfaundler, M. v., and Schlossmann, A., *Handbuch der Kinderheilkunde*, Berlin, F. C. W. Vogel, 1935, **10**, 45.

3 Lease, J. G., *Z. f. Vitaminforsch.*, 1936, **5**, 110.

4 György, P., *J. Biol. Chem.*, 1937, **119**, xliii; György, P., *J. Biol. Chem.*, 1939, **131**, 733; György, P., Kuhn, R., and Lederer, E., *J. Biol. Chem.*, 1939, **131**, 745; Birch, T. W., and György, P., *J. Biol. Chem.*, 1939, **131**, 761.

5 György, P., Proc. Soc. Exp. Biol. and Med., 1936-37, **35**, 207.

TABLE I.
Average Values Obtained in Testing the Curative Effect of Vitamin H for Egg-White Injury in Rats, by Oral and Parenteral Administration.

Preparation Tested	Source	Rat day dose administered	
		Orally, ml	Parenterally, ml
K-5	yeast	0.5	0.1
K-646	"	0.3	0.07
H-29	"	0.3	0.05
H-33	"	0.5	0.1
H-34	"	0.4	0.1
K-211	liver	0.6	0.2
K-223	"	0.75	0.15
K-554	"	0.6	0.12
H-169a	"	>0.125	0.035
H-4/35	"	0.6	0.2
H-5/35	"	1.2	0.3

fluenced by oral doses were given ½ to ¼ of the same doses parenterally with beneficial effect.

The results of the quantitative experiments are summarized in Table I.

Conclusion. The factor (vitamin H) curative of egg-white injury in rats is 3 to 5 times more effective in parenteral than in oral administration.

11100

Immediate Reactions, to Anhydrides, of Wheal-and-erythema Type.*

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Immediate skin reaction in guinea pigs sensitized to proteins were observed by Zinsser.[1] Similar reactions in pigs sensitized to protein compounds of acyl chlorides have been produced by Landsteiner and Jacobs[2] although immediate reactions to acyl chlorides themselves were not observed. The present report deals with the production of

* This investigation was aided by a grant from the Charlton Research Fund of Tufts College Medical School.

1 Zinsser, H., *J. Exp. Med.*, 1921, **34**, 495.

2 Landsteiner, K., and Jacobs, J., *J. Exp. Med.*, 1936, **64**, 625.

immediate reactions in guinea pigs, with simple chemical compounds of the hitherto neglected anhydride group.

The examination of anhydrides for capacity to sensitize guinea pigs was prompted by Kern's observation of sensitivity to phthalic anhydride in a chemist.[3] With this substance, contrary to the usual experience in similar cases, it was easy to induce definite sensitization in guinea pigs.

Following this lead, a number of anhydrides were examined. For sensitization, three or more albino guinea pigs were injected on the back twice a week, intracutaneously, with 0.05 cc of a 0.1% olive-oil solution of the respective compounds. In the case of phthalic anhydride it was necessary first to dissolve 20 mg in 0.1 cc dioxane, subsequently diluting with 1.9 cc of olive oil. Treatments were then continued for 2 or 2½ weeks, and the animals tested not more than 2 weeks after the last treatment.

Definite sensitization was observed in animals injected with citraconic, phthalic, 3-nitrophthalic, and n-caproic anhydrides. Of these compounds, citraconic anhydride gave by far the most striking results, described below.

Tests for sensitivity to citraconic anhydride were carried out on the animal's flank, from which the hair had been removed with an electric shaver, by placing a drop of a 25% solution of citraconic anhydride in dioxane on the skin and scratching through the drop, as in human scratch-tests, with a straight needle. In adequately sensitized animals a reaction appeared after a few minutes consisting of a faint to pale pink wheal ranging from several millimeters to several centimeters in diameter (Table I) with pseudopodial extensions and neighboring pinkish, elevated islands in the more reactive pigs. Reactivation of old injection-sites was also observed and the erythema was quite definite when animals were highly sensitive, being strongest 20 to 30 minutes after testing. Within an hour or two these reactions usually lost almost all color, although edema persisted until the delayed reactions developed.

Strong pink to red delayed reactions began to appear 6 to 8 hours after the test and were at their best on the following day. The occurrence of immediate reactions seems to be influenced by the interval between the last treatment and testing, and other factors, but delayed reactions were regularly present. The most striking reactions observed were several times the size of those recorded in Table I.

To determine the specificity of the above effects, guinea pigs sensitized with 3.5-dinitrobenzoyl chloride were used which gave imme-

[3] Kern, R. A., *J. Allergy*, 1939, **10**, 164.

TABLE I.

Specificity of Wheal-and-erythema Reactions to Citraconic Anhydride.

For the scratch test with citraconic anhydride a 25% dioxane solution was used; 0.1 mg of the serum compound with 3.5-dinitrobenzoyl chloride was injected intracutaneously in a volume of 0.02 c.c. Figures refer to the diameter of the lesions in millimeters.

No. of guinea pig	Treated with	Tested with: Citraconic anhydride			Tested with: 3.5-dinitrobenzoyl chloride-guinea pig serum compound		
1	Citraconic anhydride	8, pale pink, markedly elevated	7, faintly pink, elevated	8, pale pink, elevated	7, almost colorless, elevated	almost negative	2, faintly pink nodule
2		10, pale pink, markedly elevated	10, faintly pink, elevated	25, pink, swollen	5, almost colorless, sl. elevated	almost negative	2, faintly pink nodule
3		6, pink, elevated	7, pale pink, swollen	20, pink, swollen	5, colorless, almost flat	4, colorless, almost flat	2, faintly pink nodule
4	3.5-dinitro-benzoyl chloride	2, pinkness along scratch	3, pinkness along scratch	3, pinkness along scratch	9, faintly pink, markedly elevated	9, faintly pink, markedly elevated	30, faintly pink, swollen
5		2, pinkness along scratch	3, pinkness along scratch	3, pinkness along scratch	8, faintly pink, markedly elevated	10, faintly pink, markedly elevated	19, pale pink, swollen
6		2, pinkness along scratch	4, pinkness along scratch	7, pale pink, elevated	10, faintly pink, markedly elevated	14, almost colorless, swollen	35, faintly pink, swollen
7	Untreated controls	2, pinkness along scratch	2, pinkness along scratch	4, pink, slightly elevated	2, faintly pink, slightly elevated	2, faintly pink spot	2, pale pink nodule
8		1, pinkness along scratch	1, pinkness along scratch	2, pale pink, slightly elevated	5, colorless, almost flat	3, colorless, sl. elevated	2, faintly pink nodule
Read after		20 min	45 min	overnight	20 min	45 min	overnight

diate reactions to a protein-compound of this substance prepared by a method described for *p*-chlorobenzoyl chloride.[2] Cross-tests between animals sensitized respectively by 3.5-dinitrobenzoyl chloride and citraconic anhydride (Table I) demonstrated that immediate reactions occurred only to the homologous substances, and were, therefore, manifestations of specific hypersensitiveness. Neither animals sensitized to citraconic anhydride nor controls gave a reaction when tested with dioxane alone by the scratch method. The 3 animals shown in the table were selected from a batch of 8, all of which had been definitely sensitized by the treatment.

Of guinea pigs treated with other compounds of this group, those receiving phthalic anhydride became markedly sensitive, with immediate reactions of moderate size which were not easily reproducible. The delayed reactions, however, were consistently strong. Animals treated with 3-nitrophthalic anhydride gave definite delayed reactions and only occasional, slight immediate wheals with little flare. In 3 pigs treated with caproic anhydride only the delayed type of sensitization was observed except in one animal where, after 30 minutes, a faint pinkishness was present over the entire surface which the substance had touched (not solely in the vicinity of the scratch).†

Further study of the mechanism of these reactions is indicated, in regard to the possible presence of antibodies, the occurrence of immediate reactions in sensitization to simple compounds of other groups, and the special properties of anhydrides which lend themselves to sensitization of this kind.

Summary. A type of immediate wheal-and-erythema reaction, similar to that observed in human atopic hypersensitiveness, was induced in guinea pigs that had been sensitized and tested with chemical compounds of the anhydride group.

† Diffuse pinkishness appearing within half an hour was observed at the site of application of benzoyl chloride in animals sensitized to this substance, suggesting that other acyl chlorides may give similar early reactions.

11101 P

Action of Sulfanilamide *in vitro* on Aerobic Sporogenic Bacilli.

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Experiments dealing with the action of sulfanilamide and its derivatives *in vitro* were performed mostly with those pathogenic bacteria which are susceptible to the drug *in vivo,* namely: streptococci, pneumococci, meningococci, gonococci, *B. coli, B. proteus, Brucella melitensis, et al.*[1] Although there is as yet no complete agreement as to results between experiments *in vivo* and *in vitro,* the belief is growing that under both conditions the drug affects the metabolism or the reproductive mechanism of the bacteria. It seemed of interest to investigate the action of sulfanilamide on bacteria which have a more complex life cycle than those just enumerated. For this purpose, 4 strains of aerobic spore-bearing rods were chosen: 3 of the subtilis-mesentericus group and one of the *B. mycoides.* All strains were isolated in our laboratory.

As sporulation occurs more readily on solid media, a sulfanilamide agar (SA-agar) was prepared by dissolving 23 g of powdered Bacto-Nutrient agar (Difco) in one liter of aqueous solution of sulfanilamide of a desired concentration. The medium was sterilized in the usual way. Cultures of the 4 strains were prepared on SA-agar either on slants or plates and incubated at 37°C. Smears were made daily and stained with warm carbol-fuchsin solution. Cultures on plain nutrient agar (Difco) similarly treated were used as controls. Solutions of sulfanilamide 1 : 500 and 1 :1000 were arbitrarily chosen.

The macroscopic growth of the mesentericus-subtilis strains on SA-agar was approximately the same as in the control cultures while that of mycoides was scantier. Without going into details, which are irrelevant for the main purpose of this paper, we shall describe in a general way the morphology of the bacilli on SA-agar based on the microscopic study of successive daily smears made from the cultures. During the first 10 to 12 hours the growth on SA-agar develops in the same manner as on plain agar. The smears from both media reveal vegetative rod forms uniformly stained. On SA-agar, however, the rods appear longer and sometimes thicker. The *B. mycoides* shows a tendency toward thread-formation. After

[1] See Bibliography, Long, P. H., and Bliss, E. A., *The Clinical and Experimental Use of Sulfanilamide, etc.,* Macmillan, New York, 1939, p. 141.

24 to 48 hours, when the control cultures show almost exclusively naked spores and only few vegetative rods the bacilli do not sporulate at all on SA-agar. No spore formation takes place on this medium even in cultures several weeks old. Occasionally, in the first and second passage on SA-agar, few spores are present and these are very likely the so-called "dormant" spores which failed to germinate.[2] After subsequent passages through SA-agar, even these occasional spores could not be found. The suppression of spore formation by sulfanilamide was confirmed by the following experiment: Suspensions of bacilli in saline solution were prepared from week-old cultures on SA- and plain agar, both were heated at 80°C for 15 minutes to kill vegetative forms and inoculated on plain agar. The controls yielded abundant growth while subcultures from SA-agar either remained sterile or consisted of a few discrete colonies.

Marked changes occur in the morphology of the non-sporulating vegetative rods in the SA-agar cultures. On the second day of growth, the smears begin to show rods irregularly stained. In the next few days the changes are more evident. Many shadow forms with faintly stained cytoplasm appear. Some of the rods are disintegrated into a fine granular debris. Thus, during the early hours of incubation, the sulfanilamide does not appreciably interfere with the bacterial growth. The inhibitive action of the drug becomes manifest only later, after the expiration of the so-called "lag period", a finding noted by previous investigators *in vivo* and *in vitro* in connection with various other microörganisms.[1]

Cultures on SA-agar kept at room temperature for 3 to 4 weeks lose their viability and cannot be subcultured on usual media, while due to spore-formation control cultures are viable for many months.

The bacilli, after repeated passages on SA-agar, when subcultured in the usual way on plain agar, revert to their original type and show spore formation. We succeeded, however, in obtaining 4 strains of hereditary variants of asporogenous bacilli which did not form spores on ordinary media even after repeated passages. The following technic was used: A small inoculum from the bacterial growth on SA-agar was plated on plain agar and after 3 to 4 days' incubation such colonies as were proven by microscopic examination to contain no spores were selected for subculture.

In cultures on agar containing sulfanilamide 1:2000-1:4000 sporulation is not totally suspended as the smears exhibit a few spores scattered among vegetative rods. When the concentration of the

2 Burke, V., Sprague, A., and Barnes, L., *J. Infect. Diseases*, 1925, **36**, 555.

drug is lowered to 1 :8000-1 :16000 complete sporulation takes place after a retardation of 3 to 4 days.

Neoprontosil solution (one part) added to agar (4 parts) does not inhibit sporulation of the inoculated bacilli. This result can be compared with the observation of Domagk[3] who found that this drug, while effective against streptococci *in vivo,* does not inhibit their growth *in vitro.*

Since the biochemistry of the process of sporulation in general is not well known, the mechanism by which sulfanilamide suppresses it cannot be understood. The significance of a possible destruction of spore-produced catalase (Ruhle[4]) by the anticatalase properties of the oxidation products of sulfanilamide,[5] is only a matter of conjecture.

Conclusion. Sulfanilamide, when added to nutrient media, inhibits spore formation and induces degenerative changes in the bacilli of the subtilis-mesentericus-mycoides group.

11102 P

Effect of Carbon Dioxide on Glucose Metabolism of Trypanosomes.

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In the course of the study of the glucose metabolism of trypanosomes it was found that *Trypanosoma lewisi* does not decompose glucose under anaerobic conditions unless the solution contains bicarbonate. This effect has been studied during the last 4 years. Although the mechanism of the carbon dioxide action has not been established definitely, some of the main results are given in this preliminary report.

It is well known that the presence of bicarbonate is necessary for the cultivation of certain bacteria. Werkman and coworkers[1] have

[3] Domagk, G., *Deut. Med. Wochnschr.,* 1935, **61**, 250.

[4] Ruhle, G. L. A., *J. Bact.,* 1923, **8**, 487.

[5] Main, E. R., Shinn, L. E., and Mellon, R. R., Proc. Soc. Exp. Biol. and Med., 1938, **39**, 272.

[1] Wood, H. G., and Werkman, C. H., *Biochem. J.,* 1936, **30**, 48.

found that carbon dioxide is actually used up by the propionic acid bacteria, and Wood and Werkman[2] have suggested that the utilization of carbon dioxide is linked with the succinic acid production. They believe that this 4 C atom compound is formed by the addition of CO_2 to a 3 C atom compound by means of a reaction which can be considered to be the reverse of decarboxylation.

Trypanosoma lewisi also forms succinic acid from glucose; this occurs under anaerobic conditions apparently in greater proportions than with any organism or tissue so far studied. At the same time CO_2 is used up. Depending on the concentration of bicarbonate present in the system, the ratio of mols of CO_2 assimilated to mols of glucose used varies from 0.25 to 1.0. The amount of succinic acid produced under these conditions is about 1 mol or sometimes more, per 2 mols of glucose. In addition to succinic acid, lactic acid, pyruvic acid, acetic acid and traces of formic acid are produced by this organism. As stated in a previous paper[3] about 2.5 acid equivalents other than CO_2 are formed per mol of glucose used. Analysis of the acids shows that we have accounted for 90% of the total acids produced. This corresponds to slightly over 6 C atoms per molecule of glucose, suggesting assimilation of CO_2.

Glycerol does not support the life of *Trypanosoma lewisi* under anaerobic conditions even if $NaHCO_3$ is present. Under aerobic conditions this organism decomposes glycerol without formation of measurable quantities of titratable acid and with the production of CO_2. The average respiratory quotient in experiments of long duration (3 hours or more) is 0.85, suggesting complete oxidation of glycerol. Propionic acid bacteria behave differently. Wood and Werkman[2] found that they form some succinic acid even from glycerol.

If glucose is used as a substrate under aerobic conditions, *Trypanosoma lewisi* does not use up, but produces CO_2. At the same time all the other acids produced under anaerobic conditions can be found but in different proportions. The acetic acid production is increased while the succinic, pyruvic and lactic acid production is decreased. The amount of carbon in the produced titratable acids is less than that present in the glucose used. The shift from anaerobiosis to aerobiosis is thus reflected in the quantitative distribution of the acids produced, the most striking change being that the CO_2 is used under anaerobic conditions and produced under aerobic conditions. This

2 Wood, H. G., and Werkman, C. H., *Ibid.*, 1938, **32**, 1262.

3 Reiner, L., Smythe, C. V., and Pedlow, J. T., *J. Biol. Chem.*, 1936, **113**, 75.

indicates that the assimilation of CO_2 is linked with oxidation-reduction processes. Furthermore, it was found that under anaerobic conditions pyruvic acid also activates glucose metabolism of *Trypanosoma lewisi* in a similar manner but not to the same extent as CO_2. In this case pyruvic acid is used up. Carbon dioxide is not produced but the production of lactic acid is increased to approximately the same extent as the utilization of pyruvic acid. This suggests that pyruvic acid functions as a H acceptor. It is, therefore, possible that the CO_2 assimilation occurs through the reduction of CO_2 to formaldehyde and subsequent condensation of formaldehyde.

Since under aerobic and anaerobic conditions, the reactions are essentially identical, the mere difference being a shift in equilibrium of the type

$$(1)\quad \text{lactic acid} \underset{+2H}{\overset{-2H}{\rightleftarrows}} \text{pyruvic acid}$$

or

$$(2)\quad H_2CO + H_2O \underset{+2H}{\overset{-2H}{\rightleftarrows}} HCOOH \underset{+2H}{\overset{-2H}{\rightleftarrows}} CO_2$$

one would expect that CO_2 also affects the metabolism under aerobic conditions. This is difficult to demonstrate because CO_2 is quickly accumulated in the medium before quantitative measurements can be carried out. Qualitative experiments suggest, however, that bicarbonate activates glycerol under aerobic conditions.

While our results are in agreement with the assumption that the oxidation-reduction system (2) together with an enzyme acts as a H carrier and thereby activates the metabolism, it is possible that carboxylation of some intermediate is also involved.

11103 P

Effect of Antisyphilitic Treatment on Histopathology of Local Tuberculous Lesions in Syphilitic Rabbits.

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In a previous communication[1] evidence was presented indicating that untreated syphilitic rabbits react in a different manner to the intracutaneous injection of living virulent tubercle bacilli than do similarly infected nonsyphilitic rabbits.

In the syphilitic rabbits the local inflammatory reaction to the injection of tubercle bacilli was more intense, appeared earlier, was focal in character and was usually distributed about capillaries. Ulceration occurred early in these animals, was extensive and showed undermined edges. Beneath this area of ulceration epithelioid cells were relatively few, and frequently perivascular in distribution, while granulation-tissue rich in newly formed capillaries and fibroblasts was conspicuous and was followed later by dense connective-tissue formation. On the other hand, in the non-syphilitic rabbits the local inflammatory process was diffuse, bore no relationship to vascular distribution, and epithelioid cells were numerous occurring in sheets usually extending beneath and to the sides of the ulcer. Granulation-tissue was not much in evidence and there was a paucity of newly formed capillaries.

To determine whether antisyphilitic treatment modified the histopathology of local tuberculous lesions in syphilitic rabbits, 12 albino New Zealand rabbits of the same breed as that used in the earlier studies were injected intratesticularly with an emulsion of testes from a rabbit infected with the Nichols strain of *Tr. pallidum*. These animals developed typical chancres 4 weeks after inoculation, and in 8 of the rabbits metastatic lesions appeared in the opposite testes. Examination by dark field revealed numerous *Tr. pallida* in material obtained by puncture of the testes of these animals.

The rabbits were injected intravenously with 20 mg of arsphenamine per kilo of body weight 6 weeks after infection with the syphilitic virus, and one week later were again injected with 10 mg of arsphenamine per kilo of body weight. This dose or less of arsphenamine has been found by Dr. M. Severac, Dermatological Research Laboratory,

* Aided by grants from the National Research Council and the Faculty Research Committee, University of Pennsylvania, Philadelphia.

Philadelphia, to sterilize rabbits of syphilitic infection as evidenced by failure to infect normal rabbits on injection of popliteal nodes from the treated animals. Three weeks following the last injection of arsphenamine and 10 weeks after infection with the *Tr. pallida* the rabbits were infected intracutaneously at 6 widely separated points over the abdomen with 0.1 mg in 0.1 cc salt solution of the same bovine-strain tubercle bacillus as was used in the original studies. As in the previous studies, the sites of injection were removed from a series of 3 rabbits at intervals of 1, 3, 5, 12, 24, and 48 hours after the injection of tubercle bacilli. From another series of 3 rabbits the lesions were removed 3, 4, 5, 6, 7, and 14 days after infection. In a third series of 3 rabbits the lesions were removed at intervals of 3, 4, 5, 6, 7, and 8 weeks, while from a fourth series the lesions were removed at intervals of 9, 10, 11, 12, 13, and 14 weeks following infection.

The gross appearances of the local tuberculous lesions observed in the syphilitic rabbits treated with arsphenamine did not differ conspicuously from those noted in the untreated syphilitic animals. As in the untreated syphilitic rabbits, sections removed from 1 to 12 hours inclusive showed marked vascular dilatation and perivascular aggregations of mononuclear cells with large pale-staining nuclei and numerous young fibroblasts at the site of injection of tubercle bacilli. Ulceration, which was first observed 2 weeks after infection, was superficial, and was characterized by a sharply demarcated line beneath the slough, consisting of granulation-tissue rich in newly formed capillaries and young fibroblasts. As in the untreated syphilitic rabbits epithelioid cells were relatively few and were focal and not diffuse in distribution. With increasing time dense bands of connective tissue were found. The tuberculous lesions of the viscera of these rabbits killed 3 days, 12 weeks, and 8 weeks after infection with tubercle bacilli did not differ conspicuously from those seen in the untreated syphilitic rabbits. From this experiment it is concluded that local experimental tuberculosis pursues an identical course in untreated syphilitic rabbits and in syphilitic rabbits treated with treponemicidal doses of arsphenamine.

11104 P

Effect of Exercise on Growth and Cataract Development of Rats Fed Galactose.*

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The high blood galactose and galacturia always found in rats on rations containing as much as 25% galactose,[1] raises the question as to the efficiency of the utilization of this sugar for muscular work. A brief study of this problem was made possible by the loan of some revolving exercise cages. The rations were those previously reported and were fed *ad lib.*

Littermates on the 25% galactose diet were divided between stationary and exercise cages as were control animals from the respective litters on a similar diet containing glucose in place of galactose. Exercise was entirely voluntary and the number of revolutions per week was recorded. Basal caloric requirement was calculated from body weight according to the formula of Diack[2] and caloric intake from food consumed (4.19 cal. per gram).

Growth was remarkably constant and similar for all groups, male and female, with and without exercise, and showed no difference due to type of single sugar in the diet. The calculated extra calories consumed above that required for basal expenditures was similar in the 2 ration groups (65-69% for glucose, 65-71% for galactose) and was not consistently greater for the exercise rats as might have been expected. Rats on galactose rations were just as active as those on glucose rations, the extent of exercise ranging from 5,656 to 28,538 revolutions per week for 4 weeks of the experiment. All of the galactose-fed rats had developed cataract at the end of this time and the experiment with them was discontinued.

Sugar determinations indicated the same degree of galactemia (202-255 mg/100 cc) in both galactose groups regardless of exercise and a normal blood sugar (106-134 mg/100 cc) in both glucose groups.

The only difference was in the rate of cataract development which was significantly delayed in the exercise group on galactose. The litters used in this experiment were extremely susceptible showing 100% cataract on the galactose ration, but the time when lens opacities appeared in stationary cages was 20.6 days while for those in

* Contribution No. 362, Mass. State Agr. Exp. Station.

1 Mitchell, H. S., Merriam, O. A., and Cook, G. M., *J. Nutrition*, 1937, **13**, 501.

2 Diack, S. L., *J. Nutrition*, 1930, **3**, 289.

exercise cages it was 28.0 days, a difference which cannot be ignored in view of the consistency of the figures making up each average.

One must conclude from these findings that a large part of the galactose must become available either directly or indirectly for muscular and other caloric needs. Since galactose furnishes about 24% of the total calories in the diet, a failure to use any major proportion of it would be reflected in an increased food intake or a slower rate of gain—neither of which occurred. Moreover, the increment of sugar which escapes glycogenesis and accounts for that found in the blood stream is not significantly changed by exercise, but in spite of these findings, the injury to the eye is somewhat delayed when exercise is permitted.

11105

Vaginal Cycle of *Microtus guentheri* and Its Response to Estrogenic and Gonadotropic Hormones.

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Microtus guentheri, one of the Muridae is a crop destroyer and generally feared on account of its extreme fertility.

The animal matures at a comparatively early age, mating taking place on the 28th day of life. In captivity, breeding occurs throughout the year and as early as 26 days after first copulation the first litter is delivered. After 2 weeks during which time nursing of the young takes place, the mother is again capable of mating. We are indebted for these data to Prof. Bodenheimer, who stimulated us to study the sex cycle of these animals.*

The Vaginal Cycle of Microtus guentheri. Regular examinations meet with a certain amount of difficulty as these animals are accustomed to freedom and do not allow vaginal smears to be taken as easily as do albino rats and mice. If one grasps these animals by a fold of skin they tear away, shedding some of their epidermis. If one grips them by the tail, which is about 2 cm long, they shed the skin of this part too and the tail soon becomes necrotic. The most suitable method was to hold them by the limbs, but eventually this manipula-

* In 1935, Prof. Bodenheimer, Head of the Division of Entomology of the Hebrew University, Jerusalem, brought some of these animals from the Emek Yesreel (Palestine) to Jerusalem and succeeded in breeding them in his laboratory and we are indebted to him for supplying us with these experimental animals.

tion proved fatal to the animals in the course of a few months. For this reason it was impossible to keep male and female animals together and to observe the changes of the vagina existing at the time of mating. The females either died shortly from the daily manipulations for vaginal smears or they showed an aversion to mate.

We conducted experiments in 10 female animals of various ages at different seasons of the year, continuing in 2 of the animals over a period of 4 months. Even when smears were taken twice daily neither cornification nor proestrus occurred. Morphologically the vaginae were continuously in the characteristic stage of diestrus. Since the other animals kept under the same conditions but in which no vaginal smears were taken, bred rapidly and continuously, it must be assumed that in *Microtus guentheri* mating takes place when the vagina does not show typical cornification.

Vaginal Reaction of Microtus after Administration of Estrogenic Hormone. In order to ascertain whether or not the vagina of *Microtus guentheri* is able to develop the stage of cornification, we injected estrogenic hormone into 8 castrated as well as into non-castrated animals. No difference could, however, be observed in the behavior of the two groups. The reaction to estrogenic hormone (estrone or estradiolbenzoate) is shown by the following figures: 100 I. U., anestrus; 500 I. U., proestrus; 5000 I. U., estrus.

It was quite evident that *Microtus guentheri* does not respond to comparatively large doses of estrogenic hormone (100 I. U.). The stage of cornification can, however, be obtained by giving enormous doses (5000 I. U.).

Ovarian Reaction of Microtus guentheri to Administration of Gonadotropic Hormone.† For these experiments we used 29 three-week-old females in which the vagina was still closed and gave them 1-3 injections of gonadotropic hormone prepared from various sources. (a) The animals (11) proved to be unresponsive to gonadotropic hormone from pregnancy urine (10 to 2000 RU). In some instances a certain amount of follicular enlargement (which could only be ascertained by histological examination occurred after administration of 2000 RU. At no time did we find a positive vaginal smear. (b) More marked reactions were obtained with gonadotropic hormone from the blood serum of pregnant mares‡ as shown in Table I (11 animals).

Table I demonstrates that Microtus, which is otherwise rather

† We tested the preparations used in these experiments. One RU is the minimum amount of gonadotropic hormone which produces an estrous reaction on the infantile 3-weeks-old rat.

‡ We used Antex of Lovens Kemiske Fabrik, Kopenhagen.

TABLE I.

Dose of gonadotropic hormone from the serum of mare's blood	Apr. 1 Follicle maturation	Apr. 2 Follicle hemorrhage	Apr. 3 Corpus luteum formation
5 RU	—	—	—
10 RU	±	—	—
30 RU	+	—	—
100 RU	+	—	—
1000 RU	+	—	—

indifferent, responds very readily to the administration of as little as 10 RU of gonadotropic factor from mare's blood. It is, however, worthy of record to note that even the highest dosage (1000 RU) only induces follicular maturation (Apr. 1), and never the formation of follicular hemorrhage (Apr. 2) or corpus luteum (Apr. 3).

(c) The strongest reactions occurred in connection with hormone derived from animal hypophysis as shown in Table II (8 animals).

Table II shows that *Microtus guentheri* readily responds even to small doses of gonadotropic factor of pituitary origin. After administration of 4 RU follicle ripening occurred and after 8 RU corpus luteum formation took place. It is most interesting that in this animal corpus luteum formation is procured only with gonadotropic factor from hypophyseal glands and not with that derived from either mare's blood or pregnancy urine. The absence of follicle hemorrhage need not surprise us, since this reaction also occurs very rarely even in rats and guinea pigs.

Summary. In *Microtus guentheri* no vaginal cycle with a cornification stage occurs as in white mice and rats. Injections of large doses of estrogenic hormone, however, induce cornification. Gonadotropic hormone derived from different sources produces different reactions, that from pregnancy urine shows no effect, that from mare's blood serum induces follicle maturation without luteinization even in large doses, while that from animal hypophysis produces follicular maturation and corpus luteum formation. The *Microtus guentheri* may therefore be readily used as a test animal in differentiating the various types of gonadotropic hormone, especially as to their source of origin.

TABLE II.

Dose of gonadotropic hormone from animal hypophyses	Apr. 1 Follicle maturation	Apr. 2 Follicle hemorrhage	Apr. 3 Corpus luteum formation
1 RU	—	—	—
2 RU	—	—	—
4 RU	+	—	—
8 RU	+	—	+

11106

The pH of Systemic Blood in Normal and Hypertensive Dogs, Determined by Means of a Syringe Type Glass Electrode.*

Otto H. Müller and William F. Nickel. (Introduced by Bruce Webster.)

From the Department of Surgery of the New York Hospital and Cornell Medical College.

This experiment was performed in order to determine whether any pH changes of the systemic blood of dogs accompany the development of hypertension produced by partial constriction of the renal artery.[1, 2] Thirteen dogs were used; 4 were hypertensive at the beginning of the experiment; in 4 others hypertension was produced during the period of observation; the remainder served as controls. Over a period of 90 days blood was taken frequently from the saphenous vein directly into a special syringe-type glass electrode. The pH was then measured with a Beckman pH meter.

This glass electrode was a modification of the type developed by MacInnes and Belcher[3] with a capacity of 0.5 cc; it was so constructed that a sterile hypodermic needle could be attached to its tip.† The venous pressure was usually sufficient to force the blood through the straight inner tube of the electrode; occasionally slight stasis was necessary. Constant temperature of the electrode was insured by hanging it in a saturated potassium chloride solution which was kept at 37.5°C by circulating water. This potassium chloride solution served further as a liquid junction between the glass electrode and the reference half-cell. The electrode was calibrated with buffer solutions of known pH and was checked between each sample of blood. The accuracy of the measurements was 0.03 pH units.

Although only 0.5 cc of blood was necessary to fill the glass electrode completely, usually another 0.5 cc of blood was passed through it to make sure that the blood was of the same composition as that in the vein. Before taking a sample, the electrode was rinsed with a solution of heparin (obtained from Connaught Laboratories, University of Toronto, Canada, or Hynson, Westcott and Dunning, Inc.,

* Supported by a grant from the John and Mary B. Markle Foundation.

1 Goldblatt, H., Lynch, J., Hanzal, R. F., and Summerville, W. W., *J. Exp. Med.*, 1934, **59**, 347.

2 Glenn, F., and Child, C. G., *Arch. Surg.*, 1938, **36**, 373.

3 MacInnes, D. A., and Belcher, D., *Ind. Eng. Chem. Anal. Ed.*, 1933, **5**, 199.

† Details of the glass electrode will be described elsewhere.

Baltimore, Md.) in 0.9% sodium chloride. Most of this was washed out by the stream of blood, but enough remained to prevent clotting. The blood pH in the presence of heparin did not differ from that in its absence in agreement with the observations of others.[4, 5] The measurements were completed in less than one minute from the time the blood was drawn so that changes in the blood pH due to glycolysis[5] could not have occurred. Blood samples taken repeatedly from the same dog during an hour had identical pH values, demonstrating the reproducibility of the method. From day to day, however, the blood pH of individual dogs varied considerably, which may have been due largely to different degrees of excitement and activity, or to variations in the diet. In Fig. 1 the arterial blood pressures and the pH values of the systemic blood are compared for one dog in each of the 3 groups. The blood pressures were measured by means of Van Leersum carotid loops.[6, 7] It may be seen from Fig. 1 that the variations in pH and arterial pressure do not go in parallel.

The results obtained are summarized in Tables I and II. If the means are calculated for Table I we find pH 7.32 corresponding to an

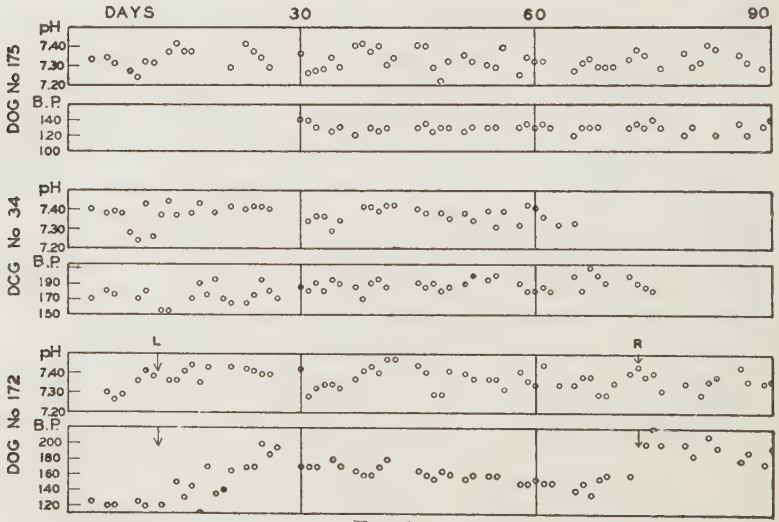

FIG. 1.

B.P.—Arterial pressure in mm mercury.
L—Application of clamp to left renal artery.
R—Application of clamp to right renal artery.

[4] Yoshimura, H., *J. Biochem. Tokyo*, 1936, **23**, 335.

[5] Haugaard, G., and Lundsteen, E., *Biochem. Z.*, 1936, **285**, 270.

[6] Van Leersum, E. C., *Arch. f. d. ges. Physiol.*, 1911, **142**, 377.

[7] Child, C. G., and Glenn, F., *Arch. Surg.*, 1938, **36**, 381.

TABLE I.

Dog No. / Sex	Normal					Hypertensive			
	3ay F	4ay F	175 F	176 M	402 M	34 M	135 M	161 M	173 M
Previous B.P. mm Hg.						195	170	180	160
Hypertensive for						30 mo.	7 mo.	6 mo.	1 mo.
Avg B.P. mm Hg.									
1st 30 days	160 (18)*	125 (18)				175 (19)	170 (18)	180 (20)	165 (18)
2nd 30 days	145 (20)	140 (20)	130 (20)			190 (22)	180 (21)	170 (22)	160 (19)
3rd 30 days	140 (19)	140 (19)	130 (18)	125 (13)		195 (19)	175 (21)	170 (19)	155 (19)
Avg pH									
1st 30 days	7.37 (15)	7.35 (15)	7.33 (16)	7.35 (15)	7.31 (15)	7.38 (18)	7.35 (16)	7.36 (15)	7.37 (21)
2nd 30 days	7.34 (24)	7.31 (22)	7.33 (25)	7.32 (22)	7.28 (24)	7.37 (21)	7.33 (25)	7.35 (25)	7.34 (21)
3rd 30 days	7.34 (7)	7.31 (5)	7.32 (19)	7.29 (12)	7.27 (22)	7.36 (4)	7.34 (5)	7.33 (7)	7.32 (9)
Total average B.P./pH	150/7.35	135/7.32	130/7.33	125/7.32	7.28	190/7.37	175/7.34	175/7.35	160/7.35

*The figures in parentheses indicate the number of observations.

TABLE II.

Dog No.	159	167	172	174
Sex	F	F	M	F
Avg B.P. before first clamp	140 (4)*	130 (5)	120 (6)	135 (30)
" " after " "	200 (4)	170 (39)	160 (43)	160 (20)
" " " second "	—	205 (7)	195 (11)	—
" pH before first "	7.30 (6)	7.34 (7)	7.33 (6)	7.38 (30)
" " after " "	7.26 (3)	7.32 (39)	7.38 (45)	7.37 (22)
" " " second "	—	7.31 (6)	7.37 (13)	—

*The figures in parentheses indicate the number of observations.

arterial blood pressure of 135 mm of mercury for the normal dogs, and a pH of 7.35 corresponding to an arterial pressure of 175 mm of mercury in the hypertensive dogs. This difference is within the experimental error of our method of measurement; however, since it is based on the average of many values, we feel that it may be significant enough to indicate the direction of pH changes in the blood due to clamping of the renal arteries. Further experiments are in progress to determine the pH changes in each renal vein.

With respect to Table II it may be mentioned that dogs 159 and 167 which had a sharp rise in arterial pressure with a change in blood pH towards more acid values, died before the experiment was finished due to uremia. The other hypertensive dogs with the more alkaline blood have a normal blood urea nitrogen content.

It may be concluded from this experiment that the buffering capacity of the systemic blood is sufficient to offset the introduction of an alkaline or acid substance if such is produced by the kidney as the result of ischemia.

11107

Bacteriostatic Actions of Three Thiazol Derivatives of Sulfanilamide upon Bacteria in Broth Cultures.

C. A. LAWRENCE. (Introduced by O. W. Barlow.)

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On the basis of *in vitro* studies 3 members of a new group of compounds, namely 2-sulfanilamidothiazol (sulfathiazol), 2-sulfanilamido-4-methylthiazol (sulfamethylthiazol), both of which were recently described by Fosbinder and Walter,[1] and 2-sulfanilamido-4-

[1] Fosbinder, R. J., and Walter, L. A., *J. Am. Chem. Soc.*, 1939, **61**, 2032.

phenylthiazol (sulfaphenylthiazol) appear to be distinctly superior to sulfanilamide and sulfapyridine in their bacteriostatic effects on pneumococci Types I, II and III, beta *Streptococcus hemolyticus* Group A, gonococci and *Staphylococcus aureus*.

Methods. Dilutions of the drugs were prepared in veal dextrose broth of pH 7.4 and containing bacto peptone. Sterile horse serum was added to the broth as an enriching substance for the pneumococci and streptococci. Ascitic fluid was used to enrich the medium for the gonococci.

To 5 cc of drug-broth solution was added one drop of an 18-hour undiluted broth culture of organisms. At the end of 5 hours' incubation at 37°C a transfer of 1 drop was made from the first tube to a second containing the same drug-broth solution, as well as to a tube containing no drug. Similar transfers were made from the second to the third tube, from the third to the fourth tube, etc., at the end of 8, 24, 32, 48, 56, and 72 hours. All tubes were incubated at 37°C for at least 96 hours before recording the final results. A protocol on the findings of one of these tests is presented in Table I.

Results. After 5 hours' exposure of one drop of Types I, II and III pneumococci in a 5 mg % concentration (1:20,000) of the thiazol-broth solutions, a drop of the treated organism suspensions failed to elicit a growth in a second tube containing the same drug-broth solution. Sulfapyridine, on the other hand, did not exhibit this same action on Types II and III even after the seventh transfer or at the end of 72 hours. The latter compound showed some degree of bacteriostasis upon Type I pneumococci, however, here again not to the extent demonstrated by the thiazol derivatives. The sulfaphenyl

TABLE I.

Bacteriostatic Action of a 5 mg % Concentration of Sulfanilamide and Some of Its Derivatives upon Type II Pneumococci in Broth.

Organism	Compound	Time intervals of transfers in hrs. 0-5	5	8	24	32	48	56	72
Type II Pneumococci	Sulfanilamide	+	+	+	+	+	+	+	+
	Control		+	+	+	+	+	+	+
	Sulfapyridine	+	+	+	+	+	+	+	+
	Control		+	+	+	+	+	+	+
	Sulfathiazol	+	—	—	—	—	—	—	—
	Control		+	+	+	—	—	—	—
	Sulfamethylthiazol	+	—	—	—	—	—	—	—
	Control		+	+	+	+	—	—	—
	Sulfaphenylthiazol	+	—	—	—	—	—	—	—
	Control		+	+	—	—	—	—	—
	Broth culture control	+	+	+	+	+	+	+	+

\+ Indicates growth.
— Indicates no growth.

Fig 1.

Bacteriostatic Actions of Sulfapyridine and Sulfanilamidothiazol Compounds upon Pneumococci and Streptococci

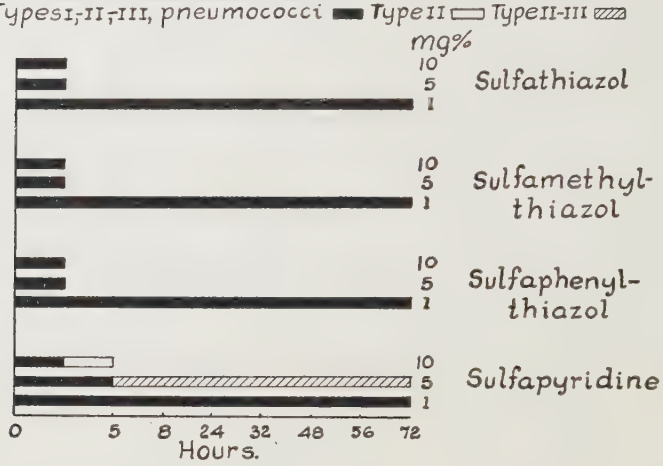
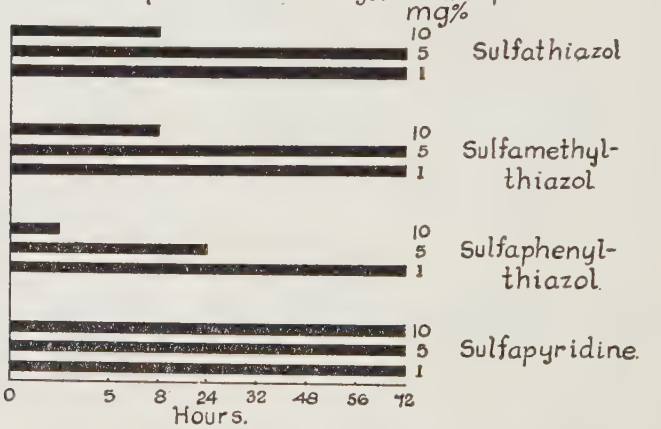


Fig 2.

Beta Streptococcus Hemolyticus Group "A"



The length of the columns for time intervals less than 72 hours indicates the time at which complete bacteriostasis occurred in the drug-broth tubes. Columns extending to 72 hours indicate growth up to this time.

compound was the only drug which presented inhibition upon the streptococci in this low concentration.

A 10 mg % concentration (1:10,000) of sulfathiazol and sulfamethylthiazol inhibited the development of streptococci in the third transfer or in the 24-hour subculture tube. The phenyl derivative was correspondingly effective in less than 5 hours; in other words, no growth was detected beyond the first inoculated tube. Sulfapyridine

produced no demonstrable effect in the same concentration throughout the entire test period. A summary of the results on pneumococci Types I, II and III and beta *Streptococcus hemolyticus,* using 1, 5, and 10 mg % concentrations of the drugs are presented in Figs. 1 and 2. The results obtained with sulfanilamide are omitted in the figures, since it was found that a 10 mg % concentration of the compound failed to produce total inhibition of any of the test organisms.

A 1 mg % concentration (1:100,000) of the 3 thiazol compounds inhibited the development of the gonococcus in the first subculture tubes, although proliferation was noted to have occurred in the first inoculated tube. Sulfapyridine in the same test inhibited growth of the organisms only at the 56-hour transfer. Sulfanilamide was ineffective throughout the entire test period. The results of this test are presented in Table II.

The action of all compounds which were effective under the conditions of these tests has been found to be purely one of bacteriostasis. These findings confirm the results obtained with the parent substance as reported by Bliss and Long,[2] Gay and his coworkers,[3] and others. By preparing dilute suspensions of the various organisms in saline and making agar plate counts, the bacteria in all instances were found to have multiplied in the first tube inoculated with the heavy broth suspension. Total inhibition, when such did occur, was first noted in the drug-broth subculture tubes. Furthermore, when bacteriostasis in the latter tubes became apparent, the corresponding control tubes containing no drug always yielded a growth in at least

TABLE II

In Vitro Effects of a 1 mg % Concentration of Sulfanilamide and Some of Its Derivatives upon Gonococci in 20% Ascitic Fluid Broth.

Organism	Compound	Time intervals of transfers in hrs.							
		0-5	5	8	24	32	48	56	72
Gonococcus Strain No. 66	Sulfanilamide	+	+	+	+	+	+	+	+
	Control		+	+	+	+	+	+	+
	Sulfapyridine	+	+	+	+	+	+	—	—
	Control		+	+	+	+	+	+	—
	Sulfathiazol	+	—	—	—	—	—	—	—
	Control		+	—	—	—	—	—	—
	Sulfamethylthiazol	+	—	—	—	—	—	—	—
	Control		+	+	—	—	—	—	—
	Sulfaphenylthiazol	+	—	—	—	—	—	—	—
	Control		+	+	—	—	—	—	—
	Broth culture control	+	+	+	+	+	+	+	+

\+ Indicates growth.
— Indicates no growth.

2 Bliss, E. A., and Long, P. H., *J. A. M. A.*, 1937, **109**, 1524.

3 Gay, Clark, Street and Miles, *J. Exp. Med.*, 1939, **69**, 607.

one transfer beyond the point at which inhibition was detected in the drug-broth solutions.

Since it was found that a 10 mg % concentration of the various compounds had little if any effect in inhibiting the growth of *Staphylococcus aureus* under the above conditions, a second method of testing these compounds on this organism was resorted to. Accurately weighed quantities of the drugs were added to 100 cc portions of veal dextrose broth. Due to the poor solubility of some of the compounds the solutions tested were, in certain instances as indicated in Table III, supersaturated. To each drug-broth solution, or solution-suspension was then added 1 cc of a dilute suspension of *Staphylococcus aureus*. All flasks containing the control and medicated media were maintained at 37°C by means of a water bath. Immediately following the addition of the inoculum, and hourly intervals thereafter, 0.1 cc of the organism-broth suspension was transferred to the center of a sterile petri dish. Melted and cooled (45°C) veal dextrose agar was then added to the plate, the contents mixed thoroughly by swirling and the agar allowed to solidify. The plates were then placed in the incubator at 37°C for 72 hours, at which time the results were recorded.

The data presented in Table III indicate that the thiazol compounds, and especially the methyl and phenyl derivatives exhibit a marked bacteriostatic action upon *Staphylococcus aureus*. Furthermore, although not indicated in the table, this inhibitory effect was conspicuous even after several days' incubation at which time interval little, if any cloudiness, indicative of growth could be detected

TABLE III.

Evaluation of Bacteriostatic Action of Sulfanilamide and Some of Its Derivatives upon *Staphylococcus aureus* by Direct Plate Counts.

Compounds	mg %	Solution	Hours						
			0	2	3	4	5	6	7
Sulfanilamide	200	Complete	11	26	70	221	387	950	1,800
	100	"	5	34	149	441	930	1,500	3,000
Sulfapyridine	200	Incomplete	13	19	45	186	349	800	1,500
	100	Complete	14	23	93	340	620	950	2,500
Sulfathiazol	200	"	10	9	32	95	183	364	660
	100	"	12	16	58	170	339	900	1,000
Sulfamethylthiazol	200	Incomplete	4	9	14	29	52	107	196
	100	Complete	10	17	49	133	287	760	1,400
Sulfaphenylthiazol	100	Incomplete	7	6	12	11	12	34	37
	50	"	10	13	15	19	19	32	47
	10	Complete	10	16	50	134	229	720	990
Broth Control	—	—	9	27	124	500	1,200	8,500	*

Figures of 500 or less represent actual number of colonies counted per plate. Values above 500 were approximated on the basis of counts of uniform fields.

*Too numerous to count.

in the inoculated flasks containing the larger quantities of the latter two compounds. Under similar conditions sulfanilamide and sulfapyridine exhibited a moderate bacteriostatic action but these compounds were distinctly inferior to the thiazol derivatives against the staphylococcus in the drug concentrations used. Under different experimental conditions Bliss and Long[4] reported that sulfanilamide and sulfapyridine were ineffective in their *in vitro* action upon *Staphylococcus aureus.*

Summary. Three new thiazol derivatives of sulfanilamide have been studied with respect to their bacteriostatic action upon microorganisms. These compounds were found to be superior to sulfanilamide and sulfapyridine in their inhibitory actions upon pneumococci Types I, II and III and beta *Streptococcus hemolyticus* Group A in concentrations as low as 5 mg %. Concentrations of 1 mg % proved the new derivatives to be more effective against the gonococcus than the parent compound and sulfapyridine. The methyl and phenyl derivatives were found to be markedly bacteriostatic for *Staphylococcus aureus.* Sulfanilamide and sulfapyridine exhibited a moderate degree of inhibition upon the latter organism.

11108

Negative Effect of Synthetic Vitamin B_6 Hydrochloride in Nutritional Deficiency in Man.*

ROBERT KARK, EUGENE L. LOZNER AND ARNOLD P. MEIKLEJOHN.
(Introduced by George R. Minot.)

From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard) of the Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Mass.

Spies and his associates[1] have recently reported observations on 4 patients with pellagra, who following treatment with nicotinic acid, riboflavin and thiamin chloride while they were taking a deficient diet, continued to complain of nervousness, insomnia, irritability, abdominal pain, weakness and difficulty in walking. These symptoms were relieved within 4 hours by a single dose of 50 mg of synthetic vitamin B_6 hydrochloride (2-methyl-3-hydroxy-4, 5-(hydroxymethyl)-pyri-

[4] Bliss, E. A., and Long, P. H., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 483.

* Aided in part by a grant given in honor of Francis Weld Peabody by the Ella Sachs Plotz Foundation.

[1] Spies, T. C., Bean, W. B., and Ashe, W. F., *J. Am. Med. Assn.*, 1939, **112**, 2414.

TABLE I.

Summary of Clinical and Hematological Findings in Six Patients with Nutritional Deficiency Disease Treated with Synthetic Vitamin B_6 Hydrochloride.

Patient	Sex	Age	Clinical diagnosis	Per day therapy prior to and during administration of synthetic Vitamin B_6 Hydrochloride: Ferrous Sulfate, g	Nicotinic Acid, mg	Riboflavin, mg	Thiamin Chloride, mg	Residual signs and symptoms prior to and during administration of synthetic Vitamin B_6 Hydrochloride
1	M	64	"Alcoholic" Pellagra	0.4	—	—	—	Dermatitis, sore tongue, sore mouth, irritability, weakness, neuritis
2	M	47	Endemic Pellagra†	0.4	100	2.5	20	"Neuritic" pains, paræsthesias, weakness, insomnia, anorexia
3	F	65	Endemic Pellagra†	0.4	500	10	10	Insomnia, weakness, anorexia, irritability
4	M	39	"Alcoholic" Pellagra†	0.4	500	2.5	100	"Neuritic" pains, paræsthesias, weakness, insomnia
5	F	49	"Idiopathic" Hypochromic Anemia	—	—	—	—	Dysphagia, weakness
6	F	53	Nutritional Macrocytic Anemia‡	0.4	500	—	10	Weakness, fatigue, dyspnea

Patient	Total amount of synthetic Vitamin B_6 Hydrochloride administered* mg: Oral	Intravenous	Duration of therapy in days	Hematological findings before and after treatment with synthetic Vitamin B_6 Hydrochloride	Red blood cells, millions/mm³	Hemoglobin, g (Sahli)	Mean corpuscular volume, cubic micra
1	350	1120	15	Before	3.45	12.24	101.1
				After	3.83	12.24	94.5
2	—	130	6	Before	4.07	14.56	103.9
				After	4.25	14.56	103.1
3	—	760	22	Before	3.40	11.23	97.6
				After	3.45	11.39	97.3
4	—	330	14	Before	3.57	12.32	100.4
				After	3.82	12.55	97
5	320	1140	21	Before	4.57	8.74	67.2
				After	4.40	8.58	65.9
6	940	—	10	Before	2.09	8.19	120
				After	1.81	7.64	118.2

*In the form of a fresh solution in 0.85% sodium chloride.

†The dermatitis presented by these patients had subsided prior to the vitamin B_6 administration.

‡This patient had a maximum reticulocyte response following injection of intramuscular liver extract.

dine) intravenously. Fouts and his coworkers[2] have reported the development of a microcytic hypochromic anemia in puppies taking a synthetic diet deficient in vitamin B_6, which was cured by the administration of this factor.

This communication records observations on the ineffectiveness of synthetic vitamin B_6 hydrochloride† administered to 6 patients with nutritional deficiency of types described in Table I, 5 of whom had very definite anemia.

Each patient on admission to the hospital was placed on a standard 3000 calorie diet containing only traces of the water soluble vitamins. Reticulocyte counts were done daily and counts of the red and the white blood cells and hematocrit and hemoglobin determinations were made every other day. The clinical and hematological findings are summarized in Table I. There was no evidence of a reticulocyte response in any case and the reticulocytes were always less than 2%.

Four of the patients received ferrous sulfate, nicotinic acid, thiamin chloride, riboflavin and ascorbic acid prior to and during vitamin B_6 therapy. These substances were given in order that a possible synergistic effect might not be overlooked. No toxic symptoms were encountered referable to vitamin B_6 administration in the doses tabulated.

In none of the 6 cases was any improvement observed either in the anemia or in the subjective symptoms and objective signs following vitamin B_6 administration.

The authors express their appreciation to Harriet MacDonald, B.S., for technical assistance.

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2 Fouts, P. J., Helmer, O. M., Lepkovsky, S., and Jukes, T. H., *J. Nutrition*, 1938, **16**, 197.

† Furnished through the courtesy of Merck and Co., Inc., Rahway, N. J.

11109 P

Serial Passage of the Human Influenza Virus in the European Hamster (*Cricetus cricetus*).

R. M. Taylor and Miklós Dreguss. (Introduced by J. H. Bauer.)

*From the State Institute of Hygiene, Budapest, Hungary.**

The study of viruses pathogenic for man is largely dependent on having an animal host suitable for experimentation. So far in the study of human influenza, ferrets and mice have proved to be best suited to this purpose, although both animals are far from being ideal. A number of others such as the hedgehog,[1] white rat, guinea pig,[2] guinea pig foetus,[3] Chinese mink, and David's squirrel[4] have been shown to be susceptible only to varying degrees. However, search for additional suitable animals is being continued by most investigators engaged in the study of viruses, and the purpose of the present communication is to report briefly the results of the attempts to infect the European hamster *(Cricetus cricetus)*† with influenza virus.

The strain of virus employed in these experiments was obtained 4 months previously from a person suffering from a rather typical attack of influenza. This virus had been identified immunologically as belonging to the group of human influenza viruses. In ferrets it produced characteristic symptoms but no lung lesions.

The following procedure was adopted: In each passage 2 hamsters were inoculated intranasally with 0.5 cc of virus suspension while under ether anesthesia. One of the animals was sacrificed on the 4th day, and 20% suspensions in broth-saline solution of the turbinates and lungs were prepared separately for subinoculation to mice and for transfer to the 2 hamsters of the succeeding passage. The second

*Supported in part by the International Health Division of the Rockefeller Foundation.

1 Stuart-Harris, C. H., *Brit. J. Exp. Path.*, 1936, **17**, 324.

2 Stuart-Harris, C. H., *Brit. J. Exp. Path.*, 1937, **18**, 485.

3 Woolpert, O. C., Gallagher, F. W., Rubenstein, L., and Hudson, N. P., *J. Exp. Med.*, 1938, **68**, 313.

4 Tang, F. F., *Brit. J. Exp. Path.*, 1938, **19**, 179.

† The *Cricetus cricetus* belongs to the order Rodentia, tribe Muridæ, sub-family *Cricetinæ*, not to be confused with the smaller golden hamster, *Cricetus auratus*, found in the Near East. It occurs in large numbers in Hungary and other parts of Europe and can easily be captured. For identification of the animals used in these experiments we are indebted to Dr. J. Éhik, Hungarian National Museum of Budapest.

hamster in each passage series was held for determination of immunity response. Blood was drawn from the heart preceding, and 2 weeks following, inoculation, and the serum was tested quantitatively for neutralizing antibodies. Thus in each passage effort was made to determine the existence of infection by the recovery of the virus in mice and by a rise in the circulating antibodies.

In the first experiment a 10% mouse lung suspension of the above-indicated virus, which had been through 2 ferret and 21 mouse passages, was used for inoculating the primary pair of hamsters. Ten serial passages were made. During the first 6 passages a mixture of turbinate and lung suspension was used for transfer and in the other 4 passages turbinate suspension alone. The virus was recovered from the turbinates of each passage hamster, and the duplicate animal held for immunity response showed a definite development of circulating antibodies. Serum from the hamsters taken before inoculation showed no neutralizing properties, even when used undiluted, but specimens taken 2 weeks after inoculation gave complete neutralization in dilutions approximating 1/250.

While the virus was frequently recovered from the lungs, it was not possible to continue passage of the infection when transfers were made from the lungs alone.

The rather marked stimulation of antibodies is indicative of a constitutional reaction, yet the animals manifested no definite symptoms. Other than congestion and slight swelling of the turbinates, the autopsies revealed nothing remarkable.

Subsequently it was found possible to infect and pass serially in hamsters a virus strain from a ferret which had not been adapted to mice. No throat washings from persons ill with influenza were available at the time, and it remains to be determined whether or not the hamster may be infected directly from human beings.

It may be added that 3 unsuccessful attempts were made to pass the virus serially in the European souslik *(Citellus citellus)*. The virus could not be recovered beyond the first passage, nor was there any stimulation of neutralizing antibodies, except to a slight degree, in the souslik receiving the primary inoculum.

11110 P

Peroxide Production by Type 3 Strains of Group A *Beta* Hemolytic Streptococci.

Faith P. Hadley and Philip Hadley.

From the Institute of Pathology, Western Pennsylvania Hospital, Pittsburgh.

Penfold[1] reported that peroxide-producing bacteria form black colonies on blood agar containing benzidine. He concluded that all pneumococci, and probably all streptococci, exhibit this reaction, but observed that some streptococcal strains gave both black and white colonies. Tunnicliff,[2] using Penfold's method, noted that streptococci from scarlet fever, giving specific opsonic tests, produced only white colonies while their dissociants, as well as cultures from erysipelas, yielded black colonies. Recently, Fuller and Maxted[3] stated that 10 Type 3 strains of *beta* hemolytic streptococci of Group A failed to reveal peroxide whereas 63 of 65 strains of other types did form it. Since 2 of their peroxide-negative Type 3 strains ("Richards" and "Robb") had been shown, in many earlier studies on sulfanilamide, highly susceptible to this drug, Fuller and Maxted concluded that the theory of Mellon and coworkers[4, 5] regarding the part played by peroxide in bacteriostasis caused by sulfanilamide was invalidated. The present report deals with the development of peroxide-forming variants from apparently peroxide-negative strains of Type 3 streptococci growing on benzidine blood agar.

Methods: Benzidine solution was prepared by grinding 0.5 g of Pfanstiehl C.P. benzidine base with 0.7 cc concentrated HCl and adding gradually 99.3 cc distilled water. This 0.5% solution was sterilized by filtration. At the time of pouring plates 0.5 cc of this solution, together with 1.0 cc of defibrinated rabbit blood, was added to 20 cc of beef-muscle or veal-heart infusion agar, pH 7.6, containing 1% Bacto-peptone, 0.5% salt and 1% sodium glycerophosphate. If the culture fails to grow on this concentration of benzidine the amount may be reduced to 0.1 cc. After a few passages on the lower concentration the amount may be increased to 0.25 cc, which is sufficient to give a positive reaction. Broth cultures of strains to be

1 Penfold, W. J., *Med. J. Australia*, 1922, **9**, 120.

2 Tunnicliff, R., *J. Inf. Dis.*, 1935, **57**, 147.

3 Fuller, A. T., and Maxted, W. R., *Brit. J. Exp. Path.*, 1939, **20**, 177.

4 Main, E. R., Shinn, L. E., and Mellon, R. R., Proc. Soc. Exp. Biol. and Med., 1938, **39**, 272.

5 Locke, A. P., Main, E. R., and Mellon, R. R., *J. Immunol.*, 1939, **36**, 183.

tested are streaked on the benzidine medium which is incubated at 37°C. Observations are made daily and plates at first showing only white colonies are incubated for 2 to 4 days and then left at room temperature.

Strains Tested: These included 6 Type 3 strains from Dr. Lancefield and designated by her: D58/36/3 (Richards); P279/7/1; D 121; S84/46/0; F 85 and H 89. Two other strains from Dr. Griffith were designated: US41 and Lewis. When received, all cultures were in mucoid phase except H89, which was smooth, and D58 and P279, which were predominantly mucoid but contained a few smooth-phase organisms.

Results: Streptococcal cultures that readily produce peroxide manifest black colonies within 24 to 48 hours at 37°C. Of the strains mentioned above all gave white colonies when observed after 48 hours. After the plates had stood at room temperature for 3 to 10 days, however, some of the white colonies had developed black papillae (secondary colonies). At first these were present in only a few of the white colonies but, as the plates aged, additional white colonies were observed to contain one or more of them. If growth was sufficiently prolonged they might overgrow the white portions of the original colony. When the entire colony was scraped from the agar the underlying medium was blackened only beneath the papillae.

When black papillae were subcultured to fresh benzidine blood agar pure black and pure white colonies developed, the former showing color in 2 to 4 days. Subculture from the papillae of some strains gave black-ringed colonies or colonies showing black arcs at the periphery. Continued selection of black colonies yielded strains that produced 100% black colonies after 24 hours' growth. While some strains required only 2 or 3 selections, others gave a pure black culture only after 6 or 7 selections. Pure black lines have remained stable for at least 2 months. The virulence for mice is less than that of the parent white strains.

Of 8 Type 3 strains tested we have obtained peroxide-positive variants from 7. Only D121 has thus far given negative results. Of these 7 strains 3 (Lewis, Richards and P279) were reported peroxide-negative by Fuller and Maxted.

Conclusions. These results demonstrate that Type 3 hemolytic streptococci produce peroxide under conditions determined by certain variability factors. This is also true of some other type strains. In manner of origin and in subsequent cultural behavior these peroxide-positive variants resemble the lactose-positive variants of *B. coli mutabile*. Peroxide formation by Type 3 strains was predicted by

Mellon,[6] who believed its apparent absence was due to temporary suppression of a special "growth phase" possessing this function. Whether suppression of peroxide formation in the white colonies is complete, or reduced to a degree not detectable by the present methods, remains a question.

11111

Microdetermination of Homocystine in Pure Solution with the Dropping Mercury Cathode.*

Adolph Stern and Eliot F. Beach. (Introduced by Icie G. Macy.)

From the Research Laboratory of the Children's Fund of Michigan, Detroit.

In a previous paper the polarographic microdetermination of cystine in globin hydrolysates was reported.[1] Under the conditions outlined for the experiment it was shown that methionine did not give a polarographic effect and was not detected by the mercury cathode. The fact that methionine does not interfere in the polarographic determination of cystine, provided its concentration is not greatly in excess of that of cystine, has been verified by Smith and Rodden.[2] A special calibration method was devised, however, to eliminate any errors in the cystine determination should interference arise from methionine or other amino acids present in the hydrolysates.[1]

Homocystine, which is derived from methionine by demethylation and oxidation, gives the sulfhydryl compound, homocysteine, upon reduction. Inasmuch as homocystine is a homologue of cystine, the behavior of these compounds should be analogous. While homocystine may be determined by the method of Okuda[3, 4] and photometrically,[5] the physiological importance of homocystine makes it desirable to have still another method for determining this substance

[6] Mellon, R. R., 1940, in press.

* A preliminary report of this paper was presented before the Division of Biological Chemistry at the Ninety-seventh Annual Meeting of the American Chemical Society at Baltimore, Md., April 3-7, 1939.

[1] Stern, A., Beach, E. F., and Macy, Icie G., *J. Biol. Chem.*, 1939, **130**, 733.

[2] Smith, E. R., and Rodden, C. J., *J. Research Nat. Bur. Standards*, 1939, **22**, 669.

[3] Okuda, Y., *J. Biol. Chem.* (Japan), 1925, **5**, 17.

[4] Brand, E., Cahill, G. F., and Block, R. J., *J. Biol. Chem.*, 1935, **110**, 399.

[5] Kassell, Beatrice, *J. Biol. Chem.*, 1935, **109**, xlix.

in low concentrations. For this reason the possible catalytic effect of homocystine at the dropping mercury cathode in the presence of ammonia, ammonium chloride and cobaltous chloride has been investigated in the hope that it might furnish the basis of a method for determining homocystine in solutions.

The homocystine used in these experiments was prepared by the method of Butz and Du Vigneaud.[6] The determinations were carried out with a standard solution of 10^{-2} M homocystine in 0.177 N hydrochloric acid. Shortly before making the measurements, 1 cc of this standard solution was added to a mixture of ammonium chloride and ammonia in a 25 cc volumetric flask and diluted so that the final concentration of the diluent was 0.1 N ammonium chloride and 0.1 N ammonia. Successively, different amounts of the standard solution of homocystine were introduced into 25 cc volumetric flasks and diluted to the mark with varying amounts of stock solutions of ammonium chloride, ammonia and cobaltous chloride to make the final concentration always the same (0.1 N ammonia, 0.1 N ammonium chloride and 10^{-2} M cobaltous chloride) in accordance with the specifications of Brdicka[7] for cystine. The polarograms for several different concentrations of homocystine were measured with the Heyrovsky polarograph. (Oxygen need not be removed from the solutions.)

It was found in the present work, as Brdicka[7] determined with several other thio-acids, that homocystine gives the polarographic effect in the presence of ammonia, ammonium chloride and cobaltous chloride. It is seen in Fig. 1 that the catalytic wave of the current voltage curve of homocystine does not exhibit the rounded maximum characteristic of cystine.[1] In this respect the homocystine current voltage curve resembles that of glutathione and of metallic ions in the presence of colloidal substances. To eliminate the possibility that impurities might cause the catalytic effect the compound was recrystallized several times from water. After many recrystallizations the homocystine still gave a characteristic catalytic wave without a rounded maximum and equimolar solutions of the compound before and after recrystallization showed the same wave heights. Homocystine, like cystine, does not give the effect except in the presence of cobaltous ions.

A calibration curve for homocystine in pure solution may be constructed by plotting wave heights against concentration, in the usual

6 Butz, L. W., and Du Vigneaud, V., *J. Biol. Chem.*, 1932-33, **99**, 135.

7 Brdicka, R., *Collect. Czechoslov. Chem. Communicat.*, 1933, **5**, 148.

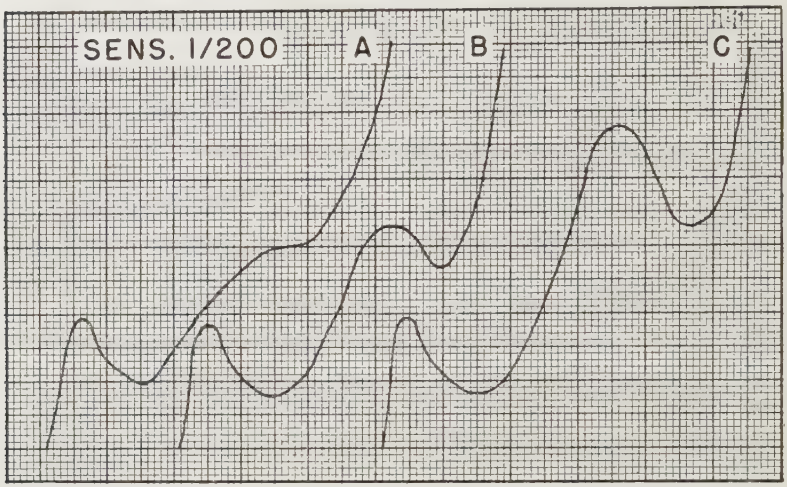

FIG. 1.

Typical current voltage curves in solutions with concentration of 0.1 *N* NH_4Cl, 0.1 *N* NH_3, and 10^{-2} *M* $COCl_2$. Curve A, homocystine. Curve B, mixture of homocystine and cystine. Curve C, cystine.

manner. By means of such a curve homocystine may be quantitatively determined in pure solutions in concentrations of only 26 γ per cc with an accuracy of ±3%. The increases in wave heights of current voltage curves of homocystine, as with cystine, are not directly proportional to the concentration.

The wave height of a solution of homocystine is somewhat lower than that of an equimolar solution of cystine. The wave heights seem to vary with the structure of the thio acids. Brdicka[7] has studied the relation of wave height to the proximity of the sulfhydryl group to the amino acid group. The wave heights of equimolar solutions of the substances studied decrease in the order thioglycolic acid, cysteyl-glycine, cysteine. The wave heights of homocysteine are lower than that of cysteine. This indicates that there is a relation between the wave heights and the proximity of the amino to the sulfhydryl group. Otherwise it is not clear why the wave height of cysteyl-glycine is higher than that of cysteine. The relations between wave height and structure, however, seem to be more complicated since the character of the thiol group is also influenced by its proximity to the carboxyl group as well as to the amino group.

Homocysteine, like homocystine, has also been found to give the catalytic wave without the rounded maximum at the dropping mercury cathode. The mechanism of the polarographic reaction of homocystine no doubt is similar to that of its lower homologue, cystine;

the homocystine is reduced to 2 molecules of homocysteine before the potential of the catalytic wave is reached. It is then homocysteine which reacts with the cobaltous ions. It is of interest that with the dropping mercury cathode homocystine is readily reduced, whereas the catalytic hydrogenation of Bergmann and Michaelis[8] which is successful when applied to cystine fails to reduce homocystine.[4] Since the polarographic effect is due to the sulfhydryl groups, the potential at which the cathode reaction takes place is practically the same for homocystine and homocysteine as it is for other thio acids such as cystine and cysteine. Therefore, it is thus far impossible to distinguish qualitatively between homocystine and cystine by the polarographic method if they are in solution together. However, in instances in which the absence of glutathione is certain, a current voltage curve without rounded maximum would be suggestive of the presence of homocystine alone.

On the other hand, an investigation of the current voltage curves given by mixtures of cystine and homocystine shows that it is possible to determine both amino acids quantitatively in the same solution. In this case the curves exhibited the rounded maximum characteristic of cystine and other thio acids (Fig. 1). Furthermore, the sum of the quantities of homocystine and cystine together, in the relative proportions thus far studied, can be calculated from the calibration curve for cystine alone and with the same accuracy. This means that the wave height due to homocystine in solution with cystine is augmented sufficiently to be almost the same as that due to an equivalent quantity of cystine sulfhydryl groups. This observation is of significance when it is recalled that homocystine in the Folin and Marenzi method[9] develops considerably less color than an equimolar amount of cystine whereas together in solution cystine and homocystine produce the full amount of color to be expected if all of the sulfhydryl groups were in the form of cystine.[4]

The polarographic method furnishes a means of determining homocystine which may be applied to biological material. Obviously it would be of value in determining methionine after quantitative demethylation. Investigations of these problems and particularly those relating to the determination of methionine are now in progress.

Summary. Homocystine and homocysteine can be determined quantitatively with the polarographic method. In concentrations as low as 26 γ per cc the accuracy is ±3%. The quantity of sulfhydryl

8 Bergmann, M., and Michaelis, G., *Ber. deutsch. chem. Ges.*, 1930, **63**B, 987, cited, *Chem. Abs.*, 1930, **24**, 3757.

9 Folin, O., and Marenzi, A. D., *J. Biol. Chem.*, 1929, **83**, 103.

groups in mixtures of homocysteine and cystine can be determined with the same accuracy although it is not possible to distinguish homocystine and cystine qualitatively by this method.

11112

Conversion of S-benzylglutathione to Benzylmercapturic Acid in the Rat.*

JACOB A. STEKOL.

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We reported recently that benzyl chloride, when administered to dogs, rabbits, and rats, yields in the urine of these animals N-acetyl-S-benzylcysteine. The same mercapturic acid was obtained from the urine of dogs, rabbits, and rats on feeding S-benzylcysteine to the animals.[1]

When S-benzylhomocysteine was administered to rabbits and rats N-acetyl-S-benzylhomocysteine was excreted in the urine.[2] This finding indicated that homocysteine is not the intermediate substance in the synthesis of benzylmercapturic acid from benzyl chloride *in vivo* and that benzylhomocysteine is not convertible to benzylcysteine in the animal body. The acetylation of S-benzyl-cysteine and S-benzylhomocysteine in the rat was confirmed.[3]

The study of the metabolism of the benzyl derivatives of sulfur-containing amino acids and their derivatives has now been extended to S-benzylglutathione. The experiments described here deal with the fate of S-benzylglutathione in the rat.

Experimental. Synthesis of S-benzylglutathione. 1.0 g of commercial glutathione was dissolved in about 30 cc of liquid ammonia and dry metallic sodium was added to the solution in small portions until a permanent blue coloration was obtained. An excess of benzyl chloride (0.5 cc) was then added dropwise, with shaking, and the ammonia was allowed to evaporate. The residue was then extracted with ether 3 times and then dissolved in water. Enough concentrated HCl was added to the solution to obtain a pH of 4 to 4.5, and

* Grateful acknowledgment is made to Mr. J. Alicino for the microanalytical work.

1 Stekol, J. A., *J. Biol. Chem.*, 1938, **124**, 129.

2 Stekol, J. A., *J. Biol. Chem.*, 1939, **128**, 199.

3 du Vigneaud, V., Wood, J. L., and Irish, O. J., *J. Biol. Chem.*, 1939, **129**, 171.

the reaction mixture was placed in a refrigerator overnight. The white granular precipitate was filtered, washed with cold water, then recrystallized twice from boiling water, filtered and dried *in vacuo* over H_2SO_4. The yield was 1.0 g, or about 80% of the theory. The analytical values of the substance are shown below.

	C	H	N	S	M.P.°C	
Found:	51.21	5.92	10.64	8.01	199-200	(uncorrected)
Calculated for $C_{17}H_{23}O_6N_3S$:	51.38	5.79	10.58	8.06		

Experiments with S-benzylglutathione. 0.4 to 0.5 g portions of S-benzylglutathione were fed mixed with food to each of 3 adult albino rats of 200 to 250 g weight. The urine was collected as was described previously.[1, 2] The urine of each rat was evaporated to a syrup *in vacuo* at 38-40°, acidified with HCl, and extracted with several portions of ethyl acetate. The extract was evaporated to dryness *in vacuo* at 38 to 40°, and the residue was dissolved in hot water. On cooling, crystalline material separated. It was centrifuged and recrystallized from hot water several times, filtered and dried *in vacuo* over H_2SO_4. The substance crystallizes from water in long needles. The analytical values are shown below.

	C	H	N	S	Acetyl	M.P.°C	
Found:	56.96	5.97	5.58	12.42	16.53	143-144	(uncorrected)
Calculated for $C_{12}H_{12}O_3NS$:	56.92	5.93	5.54	12.65	16.98		

One percent of the substance in 95% alcohol gave $[\alpha]_D^{26} = -42°$, and the melting point of 50% mixture of the substance with an authentic sample of N-acetyl-S-benzyl-*l*-cysteine remained unchanged.

The results reported above indicate the conversion of S-benzylglutathione to N-acetyl-S-benzylcysteine in the rat. It appears probable that S-benzylglutathione was hydrolyzed *in vivo* to yield S-benzylcysteine. The latter was then converted to the acetyl derivative, as could be expected from earlier work.[1, 2] As we suggested previously,[1, 2] the acetylation of S-benzylcysteine and S-benzylhomocysteine *in vivo* is probably preceded by oxidative deamination of the substances as postulated by the theory of Knoop.[4] This view is now supported by the experimental data of du Vigneaud, *et al.*[3]

It would be of interest to ascertain whether glutathione augments the synthesis of benzylmercapturic acid from benzyl chloride in the rat, particularly because our present data suggest the possibility of direct combination of benzyl chloride with glutathione *in vivo* prior

[4] Knoop, F., and Blanco, J. G., *Z. Physiol. Chem.*, 1925, **146**, 267.

to the formation of benzylmercapturic acid. It should be mentioned, however, that glutathione failed to augment the synthesis of p-bromophenyl and l-α-naphthalenemercapturic acids in the rat, suggesting the inference that glutathione is not involved directly with the synthesis of these mercapturic acids *in vivo*.[5]

Summary. 1. S-benzylglutathione was synthesized and fed to adult rats. N-acetyl-S-benzyl-*l*-cysteine was isolated from the urine of these animals and identified by analysis. 2. The results suggest that the benzylated tripeptide was hydrolyzed in the rat to yield S-benzylcysteine which was then acetylated *via* the mechanism proposed by Knoop.[4]

11113

Development of Vaccinia and Variola Viruses in Embryonated Eggs at 28°C.

JOHN B. NELSON. (Introduced by Carl TenBroeck.)

From the Department of Animal and Plant Pathology, The Rockefeller Institute for Medical Research, Princeton, N. J.

At a temperature of 28°C the embryo in fertile 10-day hen's eggs ceases to develop but may remain alive for 48 to 72 hours, showing active movement on removal. By the 4th day, however, the embryo is usually inactive and autolytic changes may be apparent in the chorioallantoic membrane. It seemed of interest to determine whether certain viruses which are readily propagated in fertile eggs at temperatures favoring a normal development of the embryo could also be maintained at 28°C.

Accordingly, observations were made on the behavior of vaccinia and variola viruses in 10-day embryonated eggs held at a constant temperature of 28°C for 2 to 4 days in a humidified incubator. The chorioallantois was retracted from the shell membrane by suction and small unmeasured amounts of the respective virus suspensions in saline were introduced through a window in the shell. The eggs were generally opened on the 3rd day and the chorioallantoic membrane was removed for microscopic examination.

Vaccinia virus originally derived from the New York City Board of Health strain was established on the chorioallantoic membrane of embryonated eggs incubated at 28°C and maintained in 2 series of

[5] Stekol, J. A., *J. Biol. Chem.*, 1938, **122**, 333.

successive transfers through 20 and 10 passages, respectively. Elementary bodies were demonstrable by the silver impregnation method of Morosow in membrane films from 47 of 60 eggs used in the first passage series and in 21 of 28 eggs in the second series. The embryo was active on the 3rd day in 27 of the virus-positive eggs of the first series and in 13 of the second. Four bacterial contaminations were encountered in the 2 series of inoculations.

The number of elementary bodies in membrane films varied considerably from egg to egg; not infrequently, however, they were as numerous as in membranes from eggs incubated at 37°C. One titration of virus was made in embryonated eggs at 37°C, using a membrane removed on the 3rd day from the 16th passage at 28°C. The titer was 10^{-6}, the membrane inoculated with this dilution showing 5 discrete foci. A second titration was carried out in the skin of a rabbit, using a 10th passage membrane at 28°C; the limiting dilution was again 10^{-6}.

The development of vaccinia virus in the chorioallantoic membrane of embryonated eggs incubated at 37°C is accompanied by pathological changes, chiefly ectodermal proliferation and necrosis, which are clearly apparent macroscopically and microscopically. With the present strain of virus the embryo is usually dead by the 3rd day.

The most striking feature of the development of vaccinia virus in embryonated eggs incubated at 28°C was the lack of reaction in the chorioallantois. Many of the membranes which contained numerous elementary bodies appeared normal macroscopically. In some instances, particularly if retraction from the shell membrane was complete, minute foci were visible. These foci were generally so small that the membrane merely appeared clouded unless examined by low power magnification which brought out their discrete nature. Membranes examined on the 3rd day rarely showed any indication of necrosis. Those examined on the 4th day often showed autolytic changes whether virus was present or not. The histological findings were likewise atypical. The mesodermal blood vessels were regularly engorged and in some membranes, chiefly those which showed macroscopic foci, there were suggestive ectodermal thickenings. In the majority of the sections, however, the ectoderm was normal and intact. The mesoderm rarely showed an infiltration of phagocytic cells. The embryo in over half of the virus-positive eggs was normal in appearance and active on the 3rd day.

A strain of variola virus isolated in 1938 and carried through 43 egg passages at 37°C[1] was established in the chorioallantois at 28°C

1 Nelson, J. B., *J. Exp. Med.*, 1939, **70**, 107.

and maintained for 8 transfers. Elementary bodies were demonstrable in the membranes from 17 of the 25 eggs employed but were usually less numerous than the elementary bodies of vaccinia. Fourteen of the virus-positive eggs contained an active embryo, in 2 of them after an incubation period of 4 days.

Most of the membranes inoculated with variola virus showed macroscopic evidence of a slight tissue reaction indistinguishable from that noted in some of the eggs inoculated with vaccinia and negligible in comparison with the reaction in membranes incubated at 37°C. At the latter temperature the development of variola virus is characterized by a focal epithelial hyperplasia which becomes confluent, resulting in a membrane many times thicker in cross-section than those incubated at 28°C. Histologically the membranes showed at most a moderate proliferation of the ectodermal cells with a few phagocytes in the mesoderm.

Summary. Vaccinia and variola viruses were established and maintained on transfer in the chorioallantoic membrane of embryonated eggs incubated at a temperature sufficiently low to prevent embryonic development (28°C). The number of elementary bodies present in the membrane on the 3rd day approximated that in eggs incubated at a temperature which favored growth of the embryo (37°C). At 28°C, however, the effect of the respective viruses on the membrane and the embryo as well as the cellular response of the former to them was significantly retarded.

11114

Identity of Natural and Synthetic Crystalline Vitamin B_6.

E. J. REEDMAN, W. L. SAMPSON AND K. UNNA. (Introduced by H. Molitor.)

From the Merck Institute of Therapeutic Research, Rahway, N. J.

Vitamin B_6 was first established as a component of the vitamin B complex by György.[1] It was subsequently isolated from natural sources by Keresztesy and Stevens,[2] Lepkovsky,[3] Kuhn and Wendt[4]

1 György, P., *Biochem. J.*, 1935, **29**, 760.

2 Keresztesy, J. C., and Stevens, J. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 64.

3 Lepkovsky, S., *Science*, 1938, **87**, 169.

4 Kuhn, R., and Wendt, G., *Ber.*, 1938, **71**, 780.

and György.[5] It was ultimately synthesized by Harris and Folkers[6] and almost simultaneously by Kuhn and others.[7] With the exception of a short note by Möller, Zima, Jung and Moll[8] in which the biological identity of the natural and synthetic products was established by their influence on the growth of bacteria there is no other information in the literature on the matter of this identity. In the course of the work on the isolation and synthesis of crystalline vitamin B_6 in the Research Laboratories of Merck & Co., Inc., we had an opportunity to investigate the activity of both the natural and synthetic materials by the single dose curative method proposed by Moll[9] as well as by the rat prophylactic procedure. A summary of these results is presented in this communication.

In the curative method 21-day-old rats were placed on a vitamin B complex free diet consisting of vitamin-free casein 18%, cerelose (dextrose) 68%, Crisco 8%, cod liver oil 2%, and O & M salt mixture 4%, supplemented with 40 micrograms each of thiamin chloride and riboflavin per rat per day.* In 5 to 8 weeks the majority of the rats developed well defined symptoms of B_6 deficiency characterized by swollen and edematous paws, thickened and sometimes encrusted ears, a denuded nose and occasional ulcerations about the mouth and tongue. When these symptoms became well pronounced a single dose of the test substance was administered orally to the rat. Its effect on the symptoms was observed over a period of 14 days and, in addition, weight records were kept on all animals since it is our experience that some significant although transient increase in weight accompanies the administration of an effective dose of B_6 to animals maintained on the above diet. Table I shows the results of the administration of various doses of natural and synthetic vitamin B_6.

Table I shows that both the synthetic and the natural vitamin B_6 are equally effective in curing the symptoms of vitamin B_6 deficiency. Furthermore, though single doses of 100 γ are sufficient to effect 100% cures in the test animals, 50 γ will completely cure 70 to 80% of the animals and cause some improvement in the remaining animals

5 György, P., *J. Am. Chem. Soc.*, 1938, **60**, 983.

6 Harris, S. A., and Folkers, K., *Science*, 1939, **89**, 347.

7 Kuhn, R., Wendt, G., Westphahl, O., and Westphahl, K., *Naturwissenschaften*, 1939, **27**, 469.

8 Möller, Zima, Jung and Moll, *Naturwissenschaften*, 1939, **27**, 288.

9 Moll, Th., and Schnittspahn, M., *E. Merck's Jahresber.*, 1938, **52**, 10.

* We recognize that this diet is deficient in other factors of the vitamin B complex but since the cure of rat acrodynia is the criterion in this test these other deficiencies do not interfere, as shown by the histopathological studies of Antopol and Unna (PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 126).

TABLE I.

Substance	Dose in γ	No. animals	No. complete cures	No. partial cures	No. no cures	Avg wt gain
Natural B_6	100	10	10	0	0	19.4
	50	6	5	1	0	13.3
	25	10	3	2	5	6.3
Synthetic B_6	100	11	11	0	0	18.8
	50	16	12	4	0	12.3
	25	7	3	3	1	9.0

and 25 γ will produce some improvement in several of the animals but very few complete cures. These data are in good agreement with those published by Moll[9] for the natural vitamin B_6.

In the rat prophylactic method 21-day-old male rats were placed on the same vitamin B complex-free diet as used in the curative test.

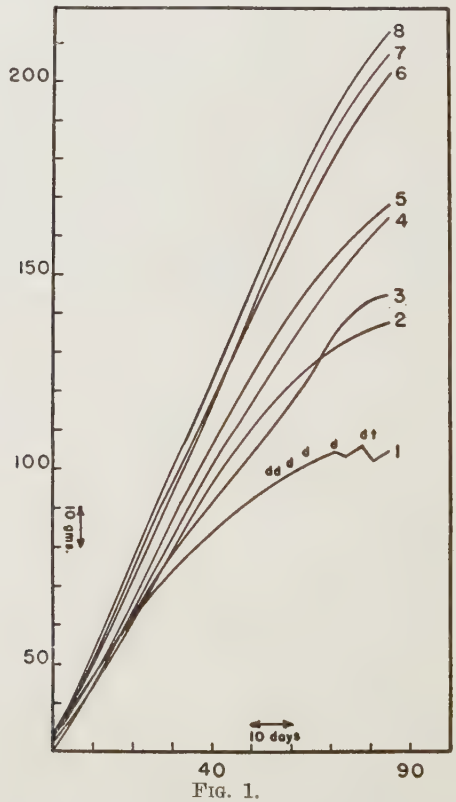


FIG. 1.

Growth response of rats to daily feeding of graded doses of natural and synthetic vitamin B_6. 1—No B_6; 2—2.5 γ synthetic B_6; 3—2.5 γ natural B_6; 4—5 γ natural B_6; 5—5 γ synthetic B_6; 6—12.5 γ synthetic B_6; 7—10 γ synthetic B_6; 8—25 γ synthetic B_6; d = dermatitis; + = death.

From the beginning of the test, each rat received a daily supplement of 40 γ each of thiamin chloride and riboflavin, 0.5 mg nicotinic acid and 0.3 cc of a highly potent liver concentrate of Factor II (Lepkovsky, Jukes and Krause[10]) kindly prepared by Dr. J. C. Keresztesy. Eight groups of 10 rats each were started on this regimen. The doses administered and the weight responses of the animals are shown in Fig. 1. The negative control group grew slowly for 7 weeks and most of the animals developed typical dermatitis within the 12 weeks' period of the test. The rats receiving 2.5 γ each of the natural and the synthetic vitamin B_6 grew at a somewhat more rapid rate but within 12 weeks their weight had plateaued, although no symptoms of dermatitis were evidenced macroscopically. The animals in these groups were sacrificed at this point. A histological examination of their tissues revealed no evidence of B_6 deficiency. The groups receiving 5 micrograms each of natural and synthetic vitamin grew more rapidly and were still gaining weight when the experiment was terminated. The rats receiving 10 micrograms, 12.5 micrograms and 25 micrograms of synthetic B_6 respectively grew at practically normal rates and there was no significant difference in growth rate among these 3 groups.

The close parallel in the rate of growth of the rats receiving 2.5 γ each of natural and synthetic B_6 and in the groups receiving 5 γ each of the natural and synthetic B_6 is further evidence as to the biological identity of these two forms of the vitamin. It is also apparent that doses greater than 10 micrograms B_6 per rat per day have no significant effect on the rate of growth. This latter observation confirms the findings of Dimick and Schreffler[11] who worked with the natural vitamin B_6 from rice polishings.

Summary. 1. Rat curative tests and rat prophylactic tests show that natural and synthetic B_6 are identical in their physiological action. 2. A single dose of 50 micrograms of either natural or synthetic B_6 is sufficient to cure severe symptoms of vitamin B_6 deficiency in 70-80% of the test animals within 14 days. 3. Ten micrograms of vitamin B_6 per rat per day is sufficient to produce practically normal growth.

We wish to acknowledge the technical assistance of Miss Josephine Dawson, Miss Evelyn Ruddy and Miss Saramae Woodford.

10 Lepkovsky, S., Jukes, T. H., and Krause, M. E., *J. Biol. Chem.*, 1936, **115**, 557.
11 Dimick, M. K., and Schreffler, C. B., *J. Nutrition*, 1939, **17**, 23.

11115 P

Toxicity of Vitamin B_6.

KLAUS UNNA AND WILLIAM ANTOPOL.

From the Merck Institute of Therapeutic Research, Rahway, N. J.

The existence of vitamin B_6, the rat acrodynia factor was first established by György.[1] The compound has since been isolated[2-5] and synthesized.[6, 7] Sufficient amounts of the synthetic vitamin, 2-methyl-3-hydroxy-4,5-dihydroxymethylpyridine, are now available for the study of the effect of large doses in animals.

The following investigation of the toxicity of the synthetic vitamin B_6 has been carried out on rats, dogs, and monkeys. All animals were maintained on completely adequate diets with the exception of one series of rats which was kept on a modified Sherman-Spohn diet supplemented with 40 micrograms of thiamin and of riboflavin per rat per day. This group developed typical symptoms of rat acrodynia.

The vitamin was administered both as the base and as the hydrochloride. The solutions of the base are neutral in reaction; those of the hydrochloride react acid, the pH of a 20% solution being 2.3.

Acute toxicity following oral and subcutaneous administration was studied in rats, 10 animals being used for each dose level. Doses up to 1 g per kg were tolerated without untoward effects. Higher doses produced rather peculiar toxic symptoms. Twenty-four hours after dosing, the rats showed tonic convulsions, the hind limbs stretched away from the body, the fore limb bent under the body and the paws closed. Between convulsive attacks the animals were able to move slowly and awkwardly and showed marked impairment of the righting reflexes. Animals receiving sublethal doses exhibited these convulsive reactions over a period extending from several days to as much as 3 weeks. With lethal doses the animals died in tonic convulsions within 36 to 72 hours. The L.D. 50* following subcutaneous injection was 3.1 g per kg for the free base and 3.7 g

1 György, P., *Biochem. J.*, 1935, **29**, 760.

2 György, P., *J. Am. Chem. Soc.*, 1938, **60**, 983.

3 Keresztesy, J. C., and Stevens, J. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 64.

4 Kuhn, R., and Wendt, G., *Ber.*, 1938, **71**, 780.

5 Lepkovsky, S., *Science*, 1938, **87**, 168.

6 Harris, S. A., and Folkers, K., *Science*, 1939, **89**, 347.

7 Kuhn, R., Wendt, G., Westphahl and Westphahl, K., *Naturwissenschaften*, 1939, **27**, 469.

* L.D. (lethal dose) 50 is the dose which is fatal to 50% of the animals.

per kg for the hydrochloride. This difference of 16% in toxicity is in good agreement with the difference of 18% in the molecular weights of the respective compounds. The L.D. 50 following oral administration was approximately 4 g per kg for the base and 6 g per kg for the hydrochloride. Autopsies of animals exhibiting various degrees of toxic manifestations failed to show pathologic changes on gross examination except for enlargement of the adrenals with occasional massive hemorrhages particularly in the cortex. Histological studies are in progress.

Three-week-old rats in groups of 15 were fed orally 0.25, 1.0, and 2.5 mg of B_6 hydrochloride daily over a period of 87 days. The animals developed normally and their weights increased at the same rate as that of the normal control group. Autopsies at the end of the 87-day feeding period showed no gross or microscopic changes in the organs. A group of 6 animals, 3 males and 3 females, were kept to maturity on a daily dose of 2.5 mg of the hydrochloride. Two litters, one of 5 and the other of 8, averaging 5 g birth weight, were obtained.

In dog studies 20 mg of B_6 per kg body weight was given orally to 3 litter mate puppies over a period of 75 days. Their weight increase was normal and did not differ from that of a control dog from the same litter. Hemoglobin, erythrocyte, leukocyte, and differential blood counts were taken at regular intervals and did not show any significant changes. Histological examination after 75 days revealed no pathological changes.

Monkeys of 2.5 and 4 kg body weight were fed or injected daily with 10 mg per kg B_6 hydrochloride. Unfortunately, most of the animals succumbed to tuberculosis during the test period. One monkey was dosed orally for 39 days, another subcutaneously for 106 days without showing any toxic symptoms. Hemoglobin and blood cell counts as well as the differential picture of the sternal bone marrow obtained by puncture did not fluctuate more than in a control monkey during the same period.†

Eight rats in severe stages of vitamin B_6 deficiency, weighing between 40 and 50 g, were given 9 mg of B_6 hydrochloride orally on two consecutive days. This dose is far in excess of the minimum curative dose of 50 micrograms.[8] Prompt cures were effected, and no toxic symptoms were observed.

† We are grateful to Dr. Lester Goldman for these data which will be presented in full in another communication.

8 Reedman, E. J., Sampson, W. L., and Unna, K., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 112.

The results of these studies characterize vitamin B_6 as a substance of very low toxicity corresponding with the low toxicity found for other members of the vitamin B complex, thiamin,[9] riboflavin,[10] and nicotinic acid.[11] Excessively large doses (3.0 g per kg) of vitamin B_6 produced convulsions and death.

The difference in subcutaneous and oral toxicity, as shown by the L.D. 50, is small and suggests a rapid and complete absorption from the intestinal tract.

Prolonged feeding of sublethal doses failed to produce toxic symptoms, thus indicating that excessive doses of vitamin B_6 are either rapidly excreted or destroyed. Studies on this problem are now in progress.

We wish to acknowledge the technical assistance of Mr. Joseph Greslin.

11116

Urinary Excretion of Vitamin B_6 in the Rat.

JOHN V. SCUDI, HAROLD F. KOONES AND JOHN C. KERESZTESY.
(Introduced by H. Molitor.)

From the Merck Institute of Therapeutic Research and Research Laboratories of Merck & Co., Inc., Rahway, N. J.

Following the isolation,[1] identification,[2] and synthesis[3] of vitamin B_6, as reported from these laboratories, the urinary excretion of the vitamin has been studied in the rat.

Recently, Kuhn and Low[4] reported on the use of the Folin-Denis reagent for the colorimetric determination of the vitamin in aqueous solutions. Stiller, Keresztesy and Stevens[2] observed that the vitamin gave a positive Gibbs reaction.[5] This reaction has been modified and

9 Molitor, H., and Sampson, W. L., *E. Merck's Jahresber.*, 1936, **50**, 51.

10 Kuhn, R., *Klin. Wchnschr.*, 1938, **17**, 222.

11 Unna, K., *J. Pharm. and Exp. Therap.*, 1939, **65**, 95.

1 Keresztesy, J. C., and Stevens, J. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 64.

2 Stiller, E. T., Keresztesy, J. C., and Stevens, J. R., *J. Am. Chem. Soc.*, 1939, **61**, 1237; Harris, S. A., and Folkers, K., *J. Am. Chem. Soc.*, 1939, **61**, 1242.

3 Harris, S. A., and Folkers, K., *J. Am. Chem. Soc.*, 1939, **61**, 1245.

4 Kuhn, R., and Low, I., *Ber.*, 1939, **72**, 1453.

5 Gibbs, H. D., *J. Biol. Chem.*, 1927, **72**, 649.

applied to the estimation of the vitamin in urine. A more detailed study of this method will be presented in the near future.

Two groups of rats were used for this investigation. One group was kept on a complete diet, the other on a diet deficient in vitamin B_6. All rats were maintained on these initial diets throughout the test period. Urine collected over the 5- to 24-hour period following the oral administration of 5 cc of water per 100 g body weight was used for control purposes. The rats were then dosed with the vitamin, accompanied by 5 cc of water per 100 g body weight, by various routes.

Urine was collected over specified periods of time. Care was exercised to prevent feces from remaining in contact with urine, since this was found to introduce interfering substances. Cages were washed into the collecting flasks to prevent urine losses, and the approximate dilution was noted. The urine samples were made strongly alkaline to thymol blue (pH above 9.6) with 30% NaOH, the volume was measured, and the sample was allowed to stand over night. This treatment suffices to destroy interfering reducing substances.

The following day 1 cc samples of the urine were neutralized to pH 7 to 7.5 and the volume was adjusted to 25, 50 or 100 cc as necessary. The adjustment of pH is of paramount importance. This is achieved by using brom thymol blue which gives a distinctive green color over the required pH range. The indicator was used externally (spot plate).

The calibration curve for the instrument was established with aqueous solutions of the vitamin at concentrations of 2 to 10 γ per cc. Urine samples were diluted to fall within this range. No interfering substances were observed when it was possible to dilute the urine samples from 1 to 50 or above. When low vitamin concentrations required a 1 to 25 dilution, occasionally interfering substances were present. These were usually negligible, although they became significant at lower dilutions. This phase of the problem is being investigated further.

Under these conditions 100% recoveries were obtained when the vitamin was added to urine.

Method. To 5 cc of the adjusted and diluted urine, 5 cc of the veronal buffer* and 20 cc of the butanol solution of the chlorimide reagent† were added.

* This was prepared by dissolving 18 g of sodium diethylbarbiturate (Merck) in 700 cc of distilled water and titrating to pH 7.6 with dilute hydrochloric acid, using the glass electrode. The solution was filtered from the precipitated barbituric acid and the pH was checked from time to time.

The tubes were briefly, but vigorously shaken, and after 5 minutes they were shaken again. After an additional 10 minutes the two layers were separated by centrifuging. The supernatant butanol layer was pipetted into 10 cc of fresh veronal solution. After shaking out extraneous colored substances‡ the two layers were separated by centrifugation, and the washing process was repeated. This treatment required less than 10 minutes. Fifteen cc of the washed butanol layer was then pipetted into a colorimeter tube which contained 5 cc of absolute ethyl alcohol, the contents were thoroughly mixed and the colors were read 40 minutes after the addition of the reagent. The colorimeter was adjusted to 100% transmission for pure butanol using a No. 660 filter. Solutions of the vitamin indophenol showed an absorption peak at 6600 Å. The color was found to be stable between 40 and 60 minutes after the addition of the reagent to the test solution.

Experimental. A series of experiments were designed to measure the total output following different modes of administration of a constant dose of 10 mg of the vitamin. Normal rats, weighing 175 to 225 g each, were placed in cages in groups of 3 or 4. Each rat was given 10 cc of water and 10 mg of vitamin B_6 and this dose was maintained daily throughout the test period of 2 weeks to insure maximum saturation of the animal. Twenty-four-hour samples of urine were analyzed, although excretion was complete in considerably less time.

Twelve rats given the vitamin intravenously (femoral vein) showed an average output of 56% (±8, maximum deviation, 12%). When administered intraperitoneally, 36 rats excreted 50% (±8, maximum deviation 14%) of the vitamin, and 48 rats put out 59% (±11, maximum deviation 24%) after oral administration of the vitamin.

The recoveries were essentially the same on the first and last days of the test. The amounts of the vitamin recovered were the same regardless of the mode of administration, thus suggesting complete

† 2,6-Dichloroquinone chlorimide (Eastman No. 2483). 100 mg were dissolved in 1600 cc of acid-free butanol and stored cold in a brown, glass-stoppered bottle. Portions withdrawn daily were allowed to warm to room temperature before use. Under these conditions the reagent is stable for at least 2 weeks. Although excellent results were obtained with the Bausch and Lomb spectrophotometer, it was thought desirable to standardize the present method for the Evelyn colorimeter. Control tests in the absence of vitamin gave 14 to 16% absorption. Solutions of the reagent which gave control readings of 20% or more were discarded.

‡ These are negligible in dilute rat urine, but are significant in other samples. These will be considered in a forthcoming communication.

absorption of the vitamin. The total average recovery of the vitamin or metabolites retaining the beta-hydroxypyridine free para position structure was 57%. The fate of the remainder has not been established, although the darkening of these urines in contrast to the stable color of the controls is significant.

A second series of 22 rats, of equal weight, were given 10 cc of water containing 1.0 mg of the vitamin orally. The average output of 67% (±7, maximum deviation 15%) is approximately equal to that obtained at the higher level.

The average output of the vitamin 5 hours after the oral administration of 36 mg of crystalline B_6 in 10 cc of water to each of 8 rats was of the same order of magnitude (71%) as that found in the 24-hour samples mentioned above. Thus it appears that the vitamin is rapidly excreted.

A group of 24 rats weighing 40 to 50 g each, was maintained on a modified Sherman-Spohn diet. When these animals showed the characteristic lesions of acrodynia, they were placed in groups of 4 for a series of 3 experiments. Eight rats were given 3 cc of water orally and 2 hours later they were given 9 mg of the vitamin in 3 cc of water orally. The urine, collected over a 5-hour period, showed a 54% recovery. This experiment repeated with the same animals the following day gave a 71% recovery. Thus, at high dose levels deficient and normal rats excrete essentially the same percentage of administered vitamin.

This experiment was repeated with a second group of 8 B_6-deficient rats, reducing the dose to 0.5 mg of the vitamin. The recovery was 65% on the first day, and 58% on the second day.

To establish differences in the output of normal and deficient rats, the remaining 8 rats were used in the same manner, except that the dose was reduced to 0.1 mg. A control group of normal rats weighing 40 to 50 g were similarly treated. Since it was not possible to dilute the urine samples sufficiently to eliminate the influence of interfering substances, quantitative data are not presented here. However, the normal rats excreted more of the vitamin than the deficient rats.

At this dose level (2 mg per kg body weight) a differentiation of normal and deficient urinary output should be possible in larger animals. Studies in the dog and human subjects are being continued along these lines.

Thanks are due to Dr. Klaus Unna for the B_6-deficient animals. The assistance of Mr. Albert Schnerring is greatly appreciated.

Summary. A colorimetric method for the determination of vita-

min B_6 has been used to study the urinary excretion of the vitamin in the rat. At high dose levels (10 mg per kg and above) 50 to 70% of the vitamin is excreted by both normal and deficient rats. The vitamin is rapidly and completely absorbed and is rapidly excreted. At low levels (2 mg per kg) the data are qualitative, but normal rats appear to excrete a higher percentage of the ingested vitamin than B_6-deficient rats.

11117

Antidermatitic Effect of Vitamin B_6 Analogues.

KLAUS UNNA. (Introduced by H. Molitor.)

From the Merck Institute of Therapeutic Research, Rahway, N. J.

The following report presents data on the vitamin activity of 10 pyridine compounds closely related to vitamin B_6 These substances were synthesized in the Research Laboratories of Merck & Co., Inc., in connection with studies on crystalline vitamin B_6.

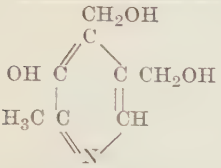
The vitamin B_6 activity of these compounds was determined by the single dose curative assay on rats, first proposed by Moll.[1] In this procedure 21-day-old rats were placed on a synthetic diet consisting of cornstarch 68%, casein 18%, Crisco 8%, salt mixture No. 1 (U.S.P. XI) 4%, cod liver oil 2%, and supplemented with 40 micrograms each of thiamin chloride and riboflavin per rat per day. After 30 days on this diet, the animals reached stationary weights and the first symptoms of dermatitis appeared. Within 7 to 10 weeks, dermatitis was fully developed in 35% of the animals. By this procedure it has been shown[2] that a single dose of 100 micrograms of vitamin B_6 cures 100% of the deficient animals within 14 days, and that a dose of 50 micrograms produces complete cures in 75% of the animals. Lower doses fail to produce complete cures, but signs of partial healing were obtained regularly with 25 micrograms and in some instances with a single dose of 15 micrograms.

In the present study 5 to 6 depleted animals were used for each dose level. Their weight and symptoms were recorded over a period of 14 days. The results obtained with the different pyridine derivatives, as

[1] Moll, Th., and Schnittspahn, M., *E. Merck's Jahresberichte*, 1938, **52**, 10.

[2] Reedman, E. J., Sampson, W. L., and Unna, K., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 112.

TABLE I.
Antidermatitic Effect of Vitamin B_6 Derivatives.

	Doses in mg						
	0.05	0.1	0.25	0.5	1.0	2.0	2.5
 2-methyl-3-hydroxy-4,5-bis (hydroxymethyl)-pyridine, Vitamin B_6	+	+					
Derivatives of 2-methyl-pyridine:							
I 3-hydroxy-4,5-bis(acetoxy-methyl)	+	+					
II 3-acetoxy-4,5-bis(acetoxy-methyl)[3]	+	+					
III 3-methoxy-4,5-bis(hydroxy-methyl)[4,5]				0	(+)		+
IV 3-hydroxy-4-methoxymethyl-5-hydroxymethyl		(+)	(+)	+			
V 3-hydroxy-4-ethoxymethyl-5-hydroxymethyl[6]		(+)	(+)	+			
VI 3-hydroxy-4,5-(epoxydimethyl)[7]		0	0	0			(+)
VII 3-hydroxy-4-methyl-5-hydroxy-methyl[8]				0	0		0
VIII 3-hydroxy-4,5-dimethyl							0
IX 3-aminoHCl-4-hydroxymethyl-5-aminomethylHCl[7]					0	0	
X 3-aminoHCl-4-ethoxymethyl-5-aminomethylHCl[7]					0	0	

+ Complete cure of at least 75% of the animals within 14 days.
(+) Partial cure accompanied by some gain in weight.
0 No curative effect and no gain in weight.

compared with the crystalline synthetic vitamin, are recorded in Table I.

The di- and triacetyl compounds (I, II) were found equally potent and of the same activity as the vitamin when equimolicular amounts were fed. The activity of these acetyl derivatives may be explained on the basis of liberation of the free vitamin by hydrolysis.

Methylation of the phenolic hydroxyl group of vitamin B_6 (III) decreased the activity to 2% of that of the vitamin. When one of

[3] Kuhn, R., and Wendt, G., *Ber.*, 1938, **71**, 780.

[4] Stiller, E. T., Keresztesy, J. C., and Stevens, J. R., *J. Chem. Soc.*, 1939, **61**, 1237.

[5] Kuhn, R., and Wendt, G., *Ber.*, 1938, **71**, 1534.

[6] Harris, S. A., and Folkers, K., *J. Am. Chem. Soc.*, 1939, **61**, 1245.

[7] Harris S. A., and Folkers, K., *J. Am. Chem. Soc.*, 1939, **61**, 3307.

[8] Harris, S. A., and Folkers, K., *J. Am. Chem. Soc.*, 1939, **61**, 1242.

the 2 hydroxymethyl groups was methylated (IV), a similar but less appreciable loss of activity was found. No difference in potency was observed between the methyl ether (IV) and the ethyl ether of the vitamin (V), but further reduction in activity resulted from the formation of an inner ether between the two hydroxyl groups in the 4 and 5 positions (VI). A derivative of VI, the lactone of 2-methyl-3-hydroxy-4-hydroxymethyl-5-carboxy pyridine,[8] has been tested in this laboratory and found to be inactive at a dose level of 1 milligram.

When either one (VII) or both hydroxymethyl groups (VIII) were replaced by a methyl group, no activity could be found at 2.5 mg. However, compound VII has been reported[9] to promote bacterial growth at a concentration 50 times higher than that of the vitamin, but to be inactive in deficient rats at a dose of 1 mg.

Introduction of amino groups in positions 3 and 5 (IX, X) resulted in inactive compounds.

Appreciation is expressed to Dr. S. A. Harris for furnishing the pyridine derivatives and to Miss J. Dawson for valuable technical assistance.

Summary. Acetylation does not diminish the antidermatitic effect of vitamin B_6. Methylation or ethylation of one of the hydroxymethyl groups diminishes the vitamin activity considerably but less than the methylation of the phenolic hydroxyl group. Replacement of one or more hydroxymethyl groups by methyl or amino groups destroys the vitamin activity.

[9] Möller, Zima, Jung and Moll, *Naturwissenschaften*, 1939, **27**, 288.

11118

Oral and Parenteral Toxicity of Vitamin K_1, Phthiocol and 2 Methyl 1, 4, Naphthoquinone.

HANS MOLITOR AND HARRY J. ROBINSON.

From the Merck Institute of Therapeutic Research, Rahway, N. J.

The antihemorrhagic activity of vitamin K_1 and its homologues has been studied by numerous investigators in animals[1–14] as well as in patients,[15–26] but no systematic investigation of the comparative toxicity of these compounds has as yet been published. Such a study would appear to be particularly indicated in view of the increasing clinical use of some of these compounds.

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1 Klose, A. A., Almquist, H. J., and Mecchi, E., *J. Biol. Chem.*, 1938, **125**, 681.

2 Dam, H., and Glavind, J., *Nature*, 1938, **142**, 1077.

3 Murphy, R., *Science*, 1938, **89**, 203.

4 Greaves, J. D., *Am. J. Physiol.*, 1939, **125**, 429.

5 Thayer, S. A., McKee, R. W., Binkley, S. B., MacCorquodale, D. W., and Doisy, E. A., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 478.

6 Ansbacher, S., *J. Nutrition*, 1939, **17**, 303.

7 Almquist, H. J., and Klose, A. A., *J. Am. Chem. Soc.*, 1939, **61**, 1611.

8 Cheney, G., *J. Lab. Clin. Med.*, 1939, **24**, 919.

9 Thayer, S. A., McKee, R. W., Binkley, S. B., MacCorquodale, D. W., and Doisy, E. A., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 194.

10 Almquist, H. J., and Klose, A. A., *J. Am. Chem. Soc.*, 1939, **61**, 1923.

11 Dam, H., and Glavind, J., *Z. Vitaminforsch.*, 1939, **9**, 71.

12 Butt, H. R., Snell, A. M., and Osterberg, A. E., *Proc. Staff Meetings Mayo Clinic*, 1939, **14**, 497.

13 Fernholz, E., Ansbacher, S., *Science*, 1939, **90**, 215.

14 Tishler, M., and Sampson, W. L., *J. Am. Chem. Soc.*, 1939, **61**, 2563.

15 Snell, A. M., Butt, H. R., and Osterberg, A. E., *Am. J. Digestive Diseases*, 1938, **5**, 590.

16 Butt, H. R., Snell, A. M., and Osterberg, A. E., *Proc. Staff Meetings Mayo Clinic*, 1938, **13**, 753.

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19 Illingworth, C. F. W., *Lancet*, 1939, **236**, 1031.

20 Scanlon, C. H., Brinkhous, K. M., Warner, E. D., Smith, H. P., and Flynn, J. E., *J. Am. Med. Assn.*, 1939, **112**, 1898.

21 Waddell, W. W., Jr., and Guerry, D., *J. Am. Med. Assn.*, 1939, **112**, 2259.

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23 Zuckerman, I. C., Kogut, B., Jacobi, M., and Cohen, J. Y., *Am. J. Dig. Diseases*, 1939, **6**, 332.

24 Townsend, S. R., and Mills, E. S., *Can. Med. Assn. J.*, 1939, **41**, 111.

25 Olson, K. B., and Menzel, H., *Surgery*, 1939, **6**, 207.

26 Koller, F., and Wuhrmann, F., *Klin. Wchnschr.*, 1939, **18**, 1058.

Acute Toxicity Studies. White mice, weighing approximately 18 to 20 g and 19-day-old chicks, weighing approximately 75 g, were used in these experiments. The compounds being practically insoluble in water, were suspended in peanut oil or sesame oil and administered orally through a blunt metal cannula to a series of 300 mice. To a second series of 240 mice and 100 chicks the drugs were given intraperitoneally in a suspension of sesame oil in order to favor a more complete absorption. In all experiments the amount of solvent given was limited to 0.25 cc of oil per 20 g animal body weight by adjusting the concentration of the suspension. In control experiments, the effect of the solvent was studied by administering peanut and sesame oil in amounts of 0.5 and 1 cc per 20 g animal body weight. Observations were made frequently during the first 5 hours and then once daily for 3 consecutive days.

The results of these experiments are shown in Charts 1 and 2. From these data it can be seen that synthetic vitamin K_1 is considerably less toxic than Phthiocol or 2-methyl-1,4-naphthoquinone when given orally or intraperitoneally. With lethal doses of Phthiocol or 2-methyl-1,4-naphthoquinone mice died usually within the first 5 hours, a few late deaths occurring on the second and third day. In contrast to this, no toxic symptoms were observed with vitamin K_1 in doses up to 25 g per kilo even over a 10-day observation period. However, in view of the fact that mice and chicks injected intraperitoneally with vitamin K_1 and sacrificed after 10 days, still had considerable amounts of the oily suspension in the abdominal cavity, the question must be raised, whether the apparent lack of toxicity of vitamin K_1 is not at least in part due to an extremely slow rate of absorption.

CHART I.

Acute Oral Toxicity of 2-methyl-1,4-naphthoquinone, Phthiocol and Vitamin K_1 in Mice.

Dose, g/kg	2-methyl-1,4-naphthoquinone	Phthiocol	Vitamin K
	% mortality		
0.100	0		
0.200	0	20	
0.300	—	70	
0.400	35	80	
0.600	50	100	
0.800	95	—	
1.000	100	—	
1.200	100	—	
1.500			
15.00			0
20.00			0
25.00			0

Total of 300 animals; 20 mice per dose level.

CHART II.

Intraperitoneal Toxicity of 2-methyl-1,4-naphthoquinone, Phthiocol and Vitamin K_1 in Mice.

Dose, g/kg	2-methyl-1,4-naphthoquinone	Phthiocol	Vitamin K_1
	% mortality		
0.050	10	0	
0.075	50	0	
0.100	90	0	
0.150	95	30	
0.200	100	100	
0.250	100	100	
0.350			
0.600			
1.000			
15.00			0
20.00			0
25.00			0

Total 240 animals, 20 mice per dose level.

Intraperitoneal Toxicity of 2-methyl-1,4-naphthoquinone, Phthiocol and Vitamin K_1 in Chicks.

Dose, g/kg	2-methyl-1,4-naphthoquinone	Phthiocol	Vitamin K_1
	% mortality		
0.10	70	50	
0.15	90	90	
0.25	100	100	
0.50	100	100	
1.00			
25.00			0

Total 100 chicks; 10 chicks per dose level on 2-methyl-1,4-naphthoquinone and Phthiocol and 20 chicks on vitamin K_1.

Chronic Toxicity. The effects of daily oral administration of vitamin K_1, Phthiocol and 2-methyl-1,4-naphthoquinone were studied in 90 young rats by feeding varying amounts of these compounds over a period of 30 consecutive days. The drugs were suspended in 10% gum acacia and doses of 0.1 and 0.35 g per kg of Phthiocol; 0.25, 0.35 and 0.5 g per kg of 2-methyl-1,4-naphthoquinone; and 0.35 and 2 g per kg of vitamin K_1 were administered by stomach tube. Gum acacia was employed in these experiments for the preparation of the suspension in order to avoid the cathartic effect likely to result from prolonged administration of oil. The weight of the animals was recorded daily and the blood picture taken in weekly intervals.

There was no significant effect upon the growth curve with any of these compounds. Rats on doses of 0.1 g per kg of Phthiocol, 0.35 g per kg of 2-methyl-1,4-naphthoquinone and 2 g per kg of vitamin K_1 appeared to remain normal whereas doses of 0.35 g per kg of Phthiocol and 0.5 g per kg of 2-methyl-1,4-naphthoquinone were lethal. Most of the animals fed toxic doses of Phthiocol died during

the first few days of the experiment while the animals fed 2-methyl-1,4-naphthoquinone died on various days over the 30-day feeding period. A marked fall of the erythrocyte count and hemoglobin was observed in rats fed doses of 0.1 g per kg of Phthiocol, and 0.35 g per kg of 2-methyl-1,4-naphthoquinone while vitamin K_1 failed to produce such an effect.

Grateful acknowledgment is made to Mr. O. Graessle and Mr. J. Mayner for valuable technical assistance.

Summary. The acute and chronic toxicity of Phthiocol, 2-methyl-1,4-naphthoquinone and vitamin K_1 was studied in mice, rats, and chicks. The oral L.D. 50 in mice was found to be approximately 0.2 g per kg for Phthiocol and 0.5 g per kg for 2-methyl-1,4-naphthoquinone; no lethal effect could be produced by doses up to 25 g per kg of vitamin K_1. In the chronic experiments in rats, daily feeding over a period of 30 consecutive days of 0.35 g per kg of Phthiocol, and 0.5 g per kg of 2-methyl-1,4-naphthoquinone was toxic; doses of 0.1 g per kg of Phthiocol and 0.35 g per kg of 2-methyl-1,4-naphthoquinone produced a marked fall of the erythrocyte count and hemoglobin. No such effects were observed following vitamin K_1 administration. In the abdominal cavity of animals sacrificed 10 days after an intraperitoneal injection of vitamin K_1 considerable amounts of an oily suspension could be observed, indicating an extremely slow rate of absorption of vitamin K_1.

11119

Effects of Culture Filtrates and Old Medium on Growth of the Ciliate, *Colpidium campylum.*

R. P. HALL AND J. B. LOEFER. (Introduced by H. W. Stunkard.)

From the Biological Laboratories, New York University and Berea College.

Effects of protozoan metabolic products on growth of homologous species have been investigated by several workers. One view, based originally upon Woodruff's[1] findings, predicates that waste products of a given species exert an inhibitory effect on growth of that species. The opposite view is represented by Dimitrowa's[2] conclusion that growth of *Paramecium caudatum* is accelerated by small amounts of old culture fluid added to fresh cultures. Johnson and Hardin[3]

[1] Woodruff, L. L., *J. Exp. Zool.*, 1911, **10**, 557.

[2] Dimitrowa, A., *Zool. Anz.*, 1932, **100**, 127.

[3] Johnson, W. H., and Hardin, G., *Physiol. Zool.*, 1938, **11**, 333.

have observed no significant effects of old culture fluid on growth of *P. multimicronucleata.* Mast and Pace,[4] however, have reported that old culture fluid in high concentration inhibits, whereas, in low concentration, it accelerates growth of *Chilomonas paramecium.* Preliminary observations, reported by Hall and Loefer,[5] indicated that growth of *Colpidium campylum* in bacteria-free cultures is significantly accelerated by the addition of old culture filtrates to a peptone medium. Kidder[6] has recently confirmed this accelerating effect of old culture fluid on growth of *C. campylum,* and has attributed it to a "biological conditioning" of the medium.

In further study of this problem, the acceleration of growth by old culture filtrates has been compared with the effects produced by aged sterile medium added to fresh peptone solution. The results obtained in 8 experimental series are described graphically in Figs. 1 and 2.

FIG. 1.

4 Mast, S. O., and Pace, D. M., *Physiol. Zool.,* 1938, **11**, 359.

5 Hall, R. P., and Loefer, J. B., *Anat. Rec.,* 1938, **72**, 50 (abstract).

6 Kidder, G. W., *Science,* 1939, **90**, 405 (abstract).

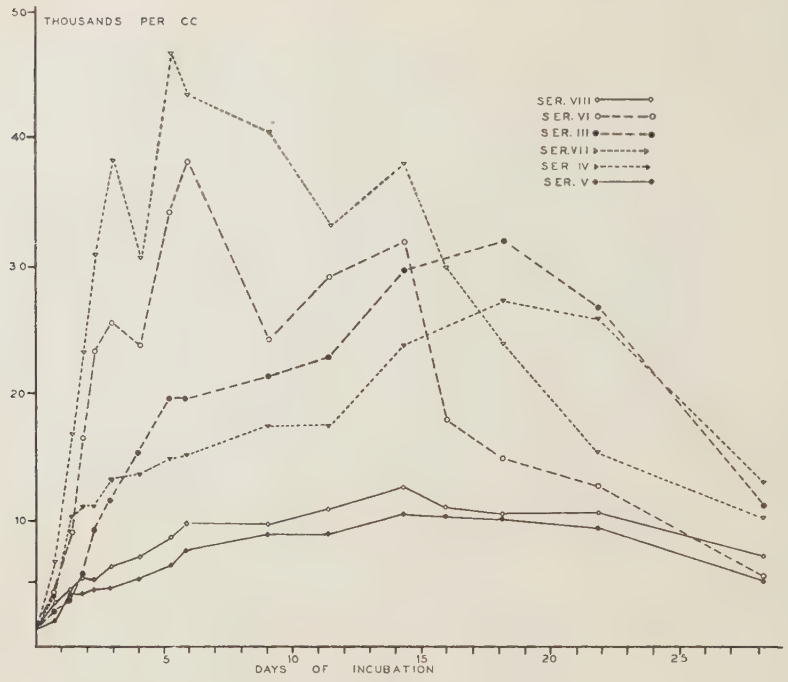

FIG. 2.

The following basic medium was used in all 8 series: casein-peptone (Difco "tryptone"), 10.0 g; KH_2PO_4, 2.0 g; glass-distilled water, 1.0 liter. However, 2 samples of tryptone were tested, one in Series I and II and the other in Series III-VIII. As indicated specifically for different series, old culture filtrate, filtered fresh medium, or filtered aged medium (uninoculated) was added to tubes containing fresh unfiltered peptone medium. In each case, Whatman No. 12 paper was used in filtration. All tubes were sterilized in the autoclave for 20 minutes at 122°C before inoculation. After inoculation, several tubes were set aside for determination of initial count (number of ciliates per cc), one tube in each series was used for initial pH readings, and the remainder were incubated as described below. Counts were made from 4 tube cultures fixed at each of the intervals indicated in Figs. 1 and 2. Our method of counting has been described elsewhere.[7] Final pH readings were taken at the end of incubation by means of a LaMotte roulette comparator.

Series I and II. In Series I, each tube contained 8.0 cc of fresh unfiltered medium and 2.0 cc of a filtrate from pooled cultures with

[7] Hall, R. P., Johnson, D. F., and Loefer, J. B., *Trans. Am. Micr. Soc.*, 1935, **54**, 298.

an average age of about 7 weeks. In Series II each tube contained 10.0 cc of unfiltered fresh medium from the same source as Series I. After sterilization, the tubes in both series received 1.0 cc inocula from the same 48-hour stock culture of *C. campylum* in peptone medium. Initial count (67 ciliates per cc), initial pH (6.1) and final pH (6.3) were the same in the two series. In both cases, cultures were incubated in darkness at room temperature.

During the first 74 hours of incubation the increase in population (Fig. 1) was somewhat more rapid in Series I. Thereafter, the differences were progressively greater until, at the end of 144 hours, the population in Series I had reached 29,353 per cc, while that in Series II was only 1,327. These results indicate that addition of old culture filtrate to fresh medium, in a dilution of 1 :5, definitely accelerates growth of *C. campylum* during the first 6 days of incubation. However, these two series were not carried long enough to determine any possible difference in maximal density of population. Furthermore, none of the medium in Series II had been passed through filter paper, and it was necessary to consider this factor in later series.

Series III-V differed in the following respects. In Series III the tubes contained 9.0 cc of fresh unfiltered medium and 1.0 cc of a 3-month-old culture filtrate; in Series IV, 9.0 cc of fresh medium and 1.0 cc of filtered, 3-month-old uninoculated medium; in the control (Series V), 9.0 cc of fresh medium (unfiltered) and 1.0 cc of filtered fresh medium. In all 3 series the tubes received 0.5 cc inocula from the same 48-hour flask culture of *C. campylum,* and the initial pH (6.3) and initial count (1219 per cc) were the same in the 3 cases. Final pH ranged from 6.9 in the controls to 7.1 in the other series. All cultures were incubated in darkness at room temperature, and counts were made at the intervals indicated.

The population curves (Fig. 2) show that, after the second day, growth was much more rapid in Series III and IV than in the control. In Series V, a rather low maximal density of population was reached about the fourteenth day, while in Series III and IV much higher maxima were reached in 18 days. Hence, it is obvious that both old culture filtrate and aged sterile medium accelerated growth of *C. campylum* and increased the density of population.

In Series VI-VIII, all tubes contained 5.0 cc of unfiltered fresh medium. In addition, the tubes in Series VI received 5.0 cc of a 3-month-old culture filtrate; those in Series VII, 5.0 cc of filtered uninoculated medium of the same age; the tubes of Series VIII (control), 5.0 cc of filtered fresh medium. Initial pH, inoculum, initial count and conditions of incubation were the same as in Series

III-V, with the exception that initial pH in Series VI was 6.5 instead of 6.3. The results are similar to those obtained in Series III-V, although growth was more rapid and maximal densities of population were higher in VI and VII than in Series III and IV. In addition, maxima were reached in 5-6 days, instead of 18 as in Series III and IV. Growth in the control (Series VIII) was similar to that in Series V. In fact, the apparent differences between the 2 controls are not statistically significant in most cases and are of doubtful significance in others. These data suggest that passage of fresh medium through filter paper added little or nothing of value to the ciliates, since the effects produced by 1.0 cc and 5.0 cc volumes are practically the same. A comparison of Series II, containing unfiltered medium only, with the other controls is not altogether valid, since initial counts were different and different samples of peptone were used in the 2 cases. While growth of the ciliates in Series II represents an increase of approximately 20 times at the end of 6 days and the corresponding increase was less than 8 times in Series V and VIII, our previous experience with large and small inocula has shown that a difference of this order would be expected even if the same sample of peptone had been used in all three series. Hence, there is no basis for assuming that mere filtration of the medium results in acceleration of growth, or that filtration removes any essential constituent of the medium.

It is interesting to note that, in spite of the difference in initial counts, the density of population in Series I after 6 days was intermediate between the corresponding densities in Series III and VI; furthermore, that the various densities were somewhat proportional to the amounts of old culture filtrate added to the medium. Thus, Series III, with 1.0 cc of filtrate, showed approximately 20,000 ciliates per cc; Series I, with 2.0 cc of filtrate, approximately 29,000; Series VI, with 5.0 cc of filtrate, approximately 38,000. A similar relationship seems to hold with respect to maximal density of population in Series III and VI; the population density showed a greater increase in the larger volume of old culture filtrate.

The effect produced by aged sterile medium was surprising. In Series IV, which received aged medium in a dilution of 1:10, growth was almost as heavy as in Series III with old culture filtrate, while the population level in Series VII was even higher than in Series VI. This is particularly interesting in view of the obvious differences between aged medium and old culture fluid. The latter had been subjected to the action of digestive enzymes and contained metabolic products liberated by the ciliates, while the changes in uninoculated

medium were undoubtedly much less extensive. Nevertheless, the mere aging of sterile medium changed it in such a way that it acquired the property of accelerating growth of *C. campylum* when added to equal or larger volumes of fresh peptone solution.

These observations have a bearing upon the question of "biologically conditioned" medium, and upon the "allelocatalytic effect" described by Robertson and others. The results obtained in Series I, III and VI might reasonably be attributed to a "biological conditioning" brought about during growth of the ciliates, if it were not for the fact that aged sterile medium produced comparable effects. Our findings do not demonstrate that the factors producing acceleration of growth are identical in old culture filtrates and in aged sterile medium, and it is possible that they are not the same. On the other hand, it is equally obvious that a "biological conditioning" cannot be invoked as the sole explanation for the accelerating effects of old culture filtrates.

Our results show further that, for studies on the "allelocatalytic effect" in ciliates, old culture fluid transferred to experimental cultures may accelerate growth in proportion to the amount of fluid carried over. Hence, in such investigations on growth in relation to initial density of population, it is imperative that the organisms in the inocula should be washed thoroughly in order to remove old culture fluid. Failure to observe such a precaution may well be responsible, at least in part, for some of the apparent conflicts in current views regarding the "allelocatalytic effect".

11120

Histological Demonstration of Vitamin A in Rats by Means of Fluorescence Microscopy.

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Vitamin A reveals a green fluorescence in ultraviolet light which disappears rapidly during irradiation.[1] This was observed under the fluorescence microscope using dilute aqueous emulsions of vitamin A concentrates. The striking green of the droplets fades during the

* Research fellow of the Cook County Graduate School of Medicine.

1 Peacock, P. R., *Lancet*, 1926, **2**, 328.

observation. The same behavior is noted in some animal tissues and may be attributed to the presence of vitamin A. Querner,[2] describing inclusions in epithelial cells of liver and adrenal, and Jancso[3] demonstrating fluorescence of the pigment epithelium of the eye, made this assumption. The assumption that such a fluorescence is due to vitamin A can be supported now by the following facts. We found this fading fluorescence always located in lipoids of the body and absent after treatment of the tissue with alcohol or acetone. Its distribution in the body agrees with that of vitamin A as determined chemically. Animal experiments, as those reported later and a few briefly mentioned by Querner offer evidence that this particular fluorescence is due to the presence of vitamin A.

Thin pieces of tissue were fixed in excess of 10% formalin and frozen sections made within 24 hours after fixation and examined under the fluorescence microscope. Inadequate and prolonged fixation must be avoided because the fatty bodies acquire a disturbing bluish fluorescence due to oxidation.

The observation of the spontaneous fluorescence of vitamin A could be supported by staining with fluorescing dyes (fluorochromy). Fats give a bluish fluorescence with methylene blue which in presence of vitamin A is surpassed by the fading green fluorescence. By changing the ultraviolet to a ground glass filter one can observe the blue stained slide in visible light and exactly localize the fluorescing inclusions. In addition, the carrier substances (lipoids) can be demonstrated by fluorescing dyes.

Twenty-three rats were grouped for vitamin A assay by Ruven Greenberg† according to U.S.P. method XI (revised 1937) and checked chemically. In 8 positive controls a regular distribution of smaller and occasionally larger droplets in the parenchyma of the liver was observed which showed a striking fluorescence fading during irradiation. The cytoplasm also revealed a green fluorescence which faded to blue. In the Kupffer cells there were also aggregations of small droplets which manifested a green fluorescence. In 7 deficient rats no green fluorescence was demonstrated; only a faint bluish fluorescence of the cytoplasm as in positive controls after fading of the green fluorescence was observed.

Six deficient animals which received more than 6600 units of vita-

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[2] Querner, F. von, *Kli. Wo.*, 1935, **14**, 1213.

[3] Jancso, N. von, and Jancso, H. von, *Biochem. Z.*, 1936, **287**, 289.

† Thanks are due to Ruven Greenberg for the preparation of the animals and the chemical determinations (method of Guilberts, H. R., and Hart, G. H., *J. Nutr.*, 1934, **8**, 25).

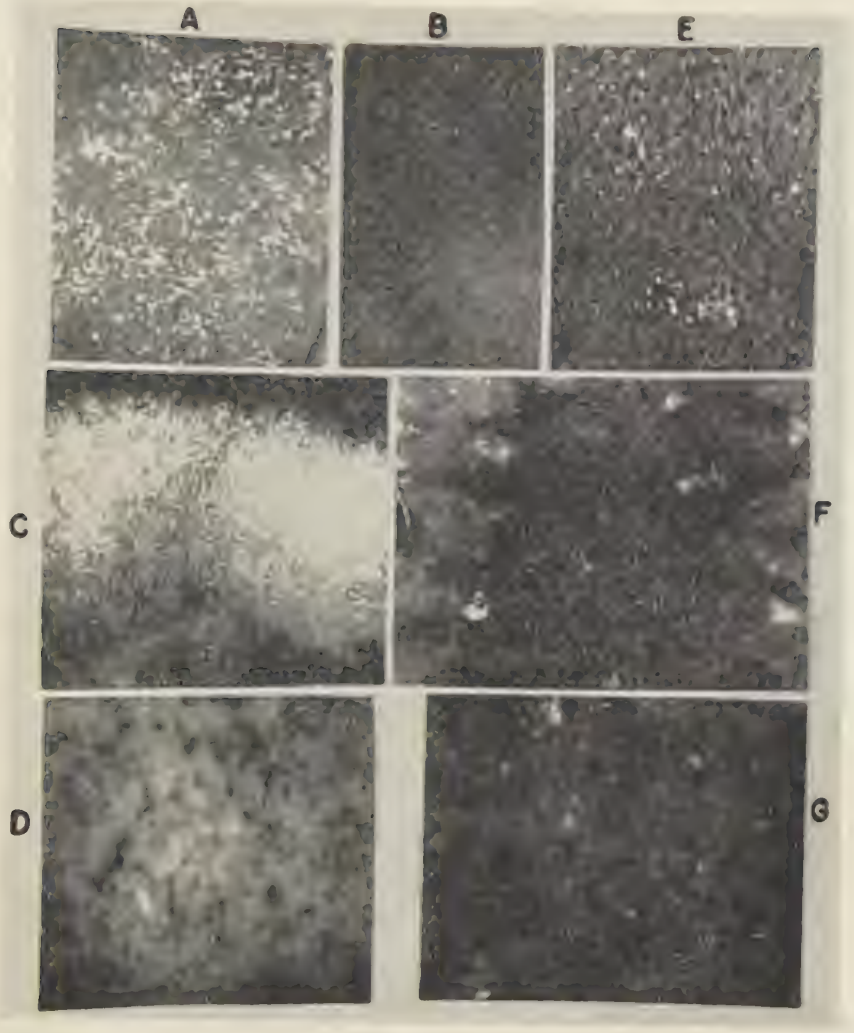

FIG. 1.

Fluorescent Microphotographs.

A. Liver of control rat. Green fading fluorescing droplets irregularly distributed in parenchyma and Kupffer cells.

B. Liver of vitamin A deficient rat.

C. Adrenal of control rat. Fine green fluorescing droplets in the cortex of the adrenal.

D. Adrenal of vitamin A deficient rat.

E. Human liver, low power. Fading green fluorescence in irregularly distributed fat droplets in liver cells, and in Kupffer cells.

F. Human liver, high power. Fading green fluorescence of Kupffer cells and of small acetone soluble droplets distributed along the edge of the epithelial cells.

G. Human liver, high power. Fading green fluorescence of Kupffer cells and the angular lipofuscin lumps which are in the center of the liver cords.

min A within sufficient time to allow storage showed many green fluorescing inclusions in epithelial and Kupffer cells, and chemically more than 1000 international units of vitamin A per gram of liver tissue. Two other animals which received 3300 and 6600 units within 3 hours before killing showed only very little green fluorescence.

The adrenals of positive controls and deficient animals after repletion revealed many small droplets with green fading fluorescence in the epithelial cells of the cortex, especially in the middle layer. In the deficient animals no such green fluorescence was present.

In the liver of 6 newborn rats there was no green fluorescence in the cytoplasm and only a few fluorescing droplets in epithelial and Kupffer cells. This is in agreement with chemical determinations of Ellison and Moore.[4] In the adrenals there was no green fluorescence.

In rats deficient in vitamin B_1 (2 rats), B_2 (2 rats), and D‡ (4 rats) normal amounts of fluorescing inclusions were found in liver and adrenal.

The liver of rabbits, monkeys, dogs, guinea pigs, mice, and frogs revealed essentially the same findings as the liver of normal rats.

11121

Production of Renin by Constricting Renal Artery of an Isolated Kidney Perfused with Blood.

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Experimental hypertension resulting from constricting the renal artery with a clamp (Goldblatt, Lynch, Hanzel and Summerville[1]) or as a result of perinephritis produced by cellophane or silk (Page[2]) is believed by many investigators to be of humoral origin and specifically to be caused by the liberation of renin from the kidneys. This renin may in turn interact with renin-activator (Kohlstaedt, Helmer

[4] Ellison, J. B., and Moore, T., *Biochem. J.*, 1937, **31**, 165.

‡ For these animals I wish to thank Dr. H. J. Cannon, Director of the Laboratory of Vitamin Technology, Chicago.

[1] Goldblatt, H., Lynch, J., Hanzal, R. F., and Summerville, W. W., *J. Exp. Med.*, 1934, **59**, 347.

[2] Page, I. H., *Science*, 1939, **89**, 273.

and Page[3]) to produce a highly active pressor substance, angiotonin (Page and Helmer[4]). It is, therefore, important to know whether renin is in fact liberated from the kidneys and if so under what circumstances.

To answer this problem it seemed desirable to employ experimental conditions as simple as possible. For this purpose isolated dogs' kidneys were perfused with blood by means of a Dale-Schuster pump.

A dog's lungs and kidneys were perfused with defibrinated blood by means of a double Dale-Schuster pump.[5] Blood flow from the renal vein was measured by a Gaddum recorder and urine flow by collection in a balanced spoon.

Samples of the renal vein blood (100 cc) were taken at intervals during perfusion of the kidney. This blood was perfused through a rabbit ear with pulsatile pressure. Injections of renin, prepared by the method of Helmer and Page[6] and renin-activator prepared according to Kohlstaedt, Helmer and Page[7] were made through a side arm in the apparatus directly into the cannula which connected the artery of the ear to the perfusion apparatus.

Forty-four experiments have been performed, typical examples of which are given.

In Experiment I, a control experiment, a kidney was perfused 240 minutes. Blood flow remained unchanged and there were only slight changes in blood pressure and urine flow (Table I). Renal vein blood collected at the beginning and at the end of the experiment was perfused through a rabbit's ear and when renin was injected into the perfusing blood, intense constriction occurred (Table II). When renin-activator was injected there was no reduction in flow.

In Experiment II after 105 minutes of perfusion a clamp was applied to the renal artery sufficiently tight to reduce pulse pressure 60%. Mean blood pressure distal to the clamp was kept constant by increasing the output of the pump. Immediately after application of the clamp blood flow was reduced 23% and there was some decrease in urine flow (Table I). In some experiments blood flow was not reduced.

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3 Kohlstaedt, K. G., Helmer, O. M., and Page, I. H., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 214.

4 Page, I. H., and Helmer, O. M., *Proc. Central Soc. Clin. Invest.*, November, 1939.

5 Dale, H. H., and Schuster, E. H., *J. Physiol.*, 1928, **64**, 356.

6 Helmer, O. M., and Page, I. H., *J. Biol. Chem.*, 1939, **127**, 757.

7 Kohlstaedt, K. G., Page, I. H., and Helmer, O. M., *Am. Heart J.*, 1940, in press.

TABLE I.
Conditions Employed During Perfusion of Kidney.

Exp. I—Without Constriction of the Renal Artery
Kidney wt 38 g.
Blood Pressure 132/92 mm Hg. at beginning of perfusion
136/86 mm Hg. after 240 min. perfusion
Mean Pressure increased from 102 mm to 111 mm Hg.
Pulse Pressure 40 mm Hg. throughout the experiment
Total Blood Flow 180 cc/min. 30 min. after perfusion started
180 cc/min. after 240 min. perfusion
Urine Flow .3 cc/min. 30 min. after perfusion started
.2 cc/min. after 240 min. perfusion
Urea Clearance 5.5 cc blood cleared per minute.
Blood Sample Taken (1) after 30 min. perfusion
(2) after 240 min. perfusion

Exp. II—With Constriction of the Renal Artery
Kidney wt 43 g
Perfused 105 min. before constricting renal artery
Blood Pressure 138/88 mm Hg.
Mean Pressure 113 mm Hg.
Pulse Pressure 50 mm Hg.
Total Blood Flow 168 cc/min.
Urine Flow 1.1 cc/min.
Urea Clearance 5.16 cc/min. blood cleared
Blood Sample Taken (3) after 30 min. perfusion; (4) 105 min. perfusion

Conditions Initiated by Application of Clamp to Renal Artery and Adjustment of Pump to Maintain Mean Pressure
Blood Pressure 120/100 mm Hg.
Mean Pressure 110 mm Hg.
Pulse Pressure 20 mm Hg. (60% reduction)
Total Blood Flow 129 cc. (23% reduction in blood flow)
Urine Flow 0.8 cc/min.

Conditions After Kidney Perfused 165 *Min. with Renal Artery Constricted*
Blood Pressure 168/164 mm Hg.
Mean Pressure 166 mm Hg. (56 mm increase)
Pulse Pressure 4 mm Hg. (16 mm decrease)
Blood Flow 76 cc/min. (41% reduction in total blood flow)
Urine Flow 0
Blood Sample (5) After 165 min. perfusion with renal artery constricted by clamp (270 min. total time perfused)

As perfusion was continued with the renal artery constricted a gradual rise in mean pressure and a further decrease in pulse pressure was observed. After 165 minutes of perfusion under these circumstances mean pressure had increased 56 mm Hg and blood flow had been reduced 41% (Table I).

When renin was injected into a rabbit ear perfused with renal vein blood taken before application of the clamp, marked vasoconstriction occurred but when the ear was perfused with blood taken 165 minutes after constriction of the renal artery, renin did not cause reduction in blood flow. Renin-activator injected into the ear perfused with blood taken before application of the clamp did not reduce

blood flow but if injected into the ear perfused with blood taken after the clamp was applied, intense vasoconstriction occurred (Table II).

These results indicate that constricting the renal artery by a clamp caused the kidney to form or liberate a substance which caused further constriction of blood vessels in the renal parenchyma.

Additional evidence favoring the view that this substance may be renin is furnished by the observation that when excess renin is added to blood in which there is no available renin-activator, vasoconstriction does not occur and if renin-activator is added to blood containing an excess of renin then vasoconstriction occurs. Blood taken prior to clamping contained renin-activator and injection of renin into the ear during perfusion with this blood, caused vasoconstriction. Blood taken after clamping apparently did not contain available renin-activator because renin injected into the ear caused no reduction in flow but it did contain a substance which resembled renin because it reacted with the renin-activator to produce vasoconstriction.

Perfusion of a dog's leg for periods similar to those employed for

TABLE II.

Effect of Injection of Renin and Renin-activator into Artery of a Rabbit's Ear During Perfusion with Defibrinated Blood Taken from the Renal Vein During Perfusion of a Kidney.

	Constriction in min.	% reduction in flow
Control Experiment.		
Exp. I—Kidney Perfused 240 Min. without applying clamp		
Blood sample (1) taken after 30 min. perfusion through kidney		
1 cc renin-activator	0	0
1 cc renin	16	91
Blood sample (2) taken after 240 min. perfusion through kidney		
1 cc renin-activator	0	0
1 cc renin	11	98
Exp. II—Clamp applied to renal artery		
Blood sample (3) taken after 30 min. perfusion and before clamp applied to artery		
1 cc renin-activator	0	0
1 cc renin	12	76
Blood sample (4) taken after 105 minutes perfusion immediately before clamp applied (Dosage of renin reduced)		
.5 cc renin-activator	0	0
.5 cc renin	3	46
.5 cc renin-activator	0	0
Blood sample (5) taken 165 min. after clamp had been applied to renal artery		
1 cc renin	0	0
1 cc renin-activator	6	75
1 cc renin	0	0
1 cc renin-activator	8	69

perfusion of the kidneys did not cause liberation of renin when the femoral artery was constricted. Renin caused vasoconstriction when injected into the femoral artery during this perfusion.

Conclusion. Renin appears to be produced by normal isolated dog's kidney perfused with blood when pulse pressure and blood flow are reduced by constricting the renal artery. Reduction of mean pressure is not a necessary condition. It is not produced when the hind leg is perfused under similar circumstances nor by kidneys perfused by blood under normal pressure-flow conditions.

11122 P

Diminution of Acetylcholine Content of Retina After Prolonged Functional Disuse.

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With aseptic precaution, the eyelids of one of the dog's eyes were sutured together, the other being left untouched as a control. After a period of blindfolding, the lids were opened up under ether anesthesia, the eyeball enucleated, and the retina carefully freed from the ciliary muscle and extracted with alcohol. All these procedures were done in the dark room under red light. The control eye was similarly prepared under ordinary illumination. The acetylcholine (AC) was identified by different tests, and the quantity determined by assay with the toad's rectus preparation.

While the AC content of the retina of the eye blindfolded for 7-49 days was found to be 11-25% less than that of the control in 5 experiments, that of the eye blindfolded for 160-170 days was over 58% less than the control in 4 experiments. The variation of the AC content of the retinae of the 2 normal eyes encountered in 12 experiments was $9.3 \pm 8.4\%$.

As there was no sign of infection, injury or irritation of any sort in the blindfolded eye, the diminution of the AC content after 160-170 days' blindfolding was apparently due to the prolonged functional disuse.

11123

Resistance of Sperm of *Rana pipiens* to Hydrostatic Compression; Effect upon Embryonic Development.

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From Washington Square College, New York University.

Various chemical and physical treatments have been applied to sperm, but only one of these, namely, radiation by X-ray or radium, has been very effective in inducing developmental changes after normal eggs have been fertilized with the treated sperm (Rugh[1]). Agents such as alcohol and other toxic substances, or such as abnormally low or high temperatures (Dunlay[2]) are difficult to use. Apparently the motility of the sperm is abolished by degrees of treatment which are insufficient to produce any marked changes in the nuclear complex of the gametes.

Three considerations suggested the use of hydrostatic pressure in an attempt to induce nuclear changes in the sperm: (1) No previous studies of the effects of pressure upon sperm have been reported; (2) It is known that high pressure can induce changes in the specificity of complex proteins such as viruses (Basset, Nicolan and Macheboeuf[3]) and antigens (Basset, Macheboeuf and Perez[4]) and therefore might conceivably be effective in inducing genic changes; and (3) In some previous work (Marsland and Brown,[5] and Pease and Kitching[6]) it has been observed that the motility of ciliated and flagellated forms is not abolished at pressures up to 600 atmospheres.

The sperm of *Rana pipiens* were obtained by excising the testes of recently hibernating frogs. Four to 6 of the intact organs, each from a different frog, were placed in a small (about 3 cc) glass test tube completely filled with 10% Holtfreter solution so as to exclude all undissolved air from the system. A rubber membrane served to seal the tube and to transmit the pressure from the surrounding Holtfreter solution which filled the bomb. To eliminate the possibility of toxic effects emanating from the rubber, the membrane was washed for a half hour in warm N/10 NaOH solution and rinsed

1 Rugh, Roberts, *Proc. Am. Phil. Soc.*, 1939, **81**, 447.

2 Dunlay, Neil S., *Biol. Bull.*, 1913, **25**, 213.

3 Basset, J., Nicolan, S., and Macheboeuf, M. A., *C. r. Acad. Sci.*, 1935, **200**, 1882.

4 Basset, J., Macheboeuf, M. A., and Perez, J. J., *C. r. Acad. Sci.*, 1935, **200**, 4960.

5 Marsland, D. A., and Brown, D. E. S., *J. Cell. and Comp. Physiol.*, 1936, **8**, 167.

6 Pease, D. C., and Kitching, J. A., *J. Cell. and Comp. Physiol.*, 1939, **14**, 135.

thoroughly in distilled water. Controls indicated that rubber so treated was non-toxic. In one experiment the sperm were liberated into the Holtfreter solution prior to the compression.

After compression, the sperm were used immediately to fertilize a minimum of 800 eggs which were obtained by the method of Rugh.[7] In most cases control fertilizations, utilizing sperm from the opposite testes of the same frogs, were carried out. The maximum available pressure, namely, 544 atmospheres (8000 lb/in.^{2}) was used in all of the experiments. The development of both the control and the experimental specimens was followed for 10 days, *i. e.*, until well after the hatching of the tadpoles.

The experimental results indicate that the sperm are quite immune to the pressures employed. In all cases they were normally motile after the compression period. Also the sperm retained their motility during the compression period, as could be seen when they were examined in the microscope-pressure chamber (Marsland and Brown[5]). There was no observable change in motility as pressure was being applied, and activity continued unabated during a compression period of 3 hours at 544 atmos. Apparently the motility of sperm is much less susceptible to the effects of pressure than is amoeboid movement (Marsland and Brown[5]), and protoplasmic streaming (Marsland[8]).

The compressed sperm gave the same fertilization results as did the controls (*i. e.*, practically 100%), and fertilization was followed by normal development well beyond the hatching stage. In the most prolonged experiment, the maximum pressure of 544 atms. was maintained continuously for a 3-hour period. Careful examination of the tail tips of tadpoles showed no appearance of haploid or other chromosome aberrations such as were obtained by X-ray treatment (Rugh[1]). A cytological study of the cells of some early embryos failed to reveal any abnormalities.

One experiment was done to determine if frequent rapid changes in the pressure level might have a greater effect than a continuously maintained high pressure. In this case during the last 2 hours of a 3-hour compression period, the pressure was dropped to 1 atms. at 5-minute intervals, and then immediately restored to 544 atms. The decompression occurred in less than 1 second and the subsequent build-up required about 5 seconds. Thus the total compression time was reduced by less than 3 minutes during the 24 successive decompressions. Nevertheless these sperm gave rise to a perfectly normal set of embryos and larvae.

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[7] Rugh, R., *Biol. Bull.*, 1935, **66**, 22.

[8] Marsland, D. A., *J. Cell. and Comp. Physiol.*, 1939, **13**, 23.

The suitability of pressure as a means of inducing genic or chromosomal changes in the sperm cannot be decided until experiments are done using higher pressures. However, pressure higher than 544 atms. is not available at present in our laboratory.

Possibly genic or chromosomal changes were not to be expected in the range of pressure which was used, despite the relatively long duration of the compression period. However, certain viruses do begin to undergo denaturation at a somewhat higher pressure, namely, 1000 atms., applied for 45 minutes (Basset, Wollman, Machelboeuf and Bardach[9]). On the other hand, a number of proteins retain their antigenic specificity at pressures up to 4000 atms. (Basset, Macheboeuf and Perez[4]) and a few do not appear to be denatured below 10,000 atms. (Basset, Lisbonne and Macheboeuf[10]).

Conclusion. Hydrostatic pressure of 544 atms. applied to frog sperm, either continuously, or in the alternating periods of compression and decompression, for a period of 3 hours, in no way alters the motility or the fertilizing power of the sperm. More than 5000 embryos resulting from such sperm and normal eggs, developed normally in all respects.

11124

Induction of Lymphomatosis in Mice Following Painting with 9:10 dimethyl-1:2 benzanthracene.

L. W. LAW* AND MARJORIE LEWISOHN.† (Introduced by C. C. Little.)

From the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine.

There is sufficient evidence to indicate the existence of a direct relationship between carcinogenic agents and certain types of tumors in mice, *e. g.*, carcinoma of the lung, sarcoma, epithelioma. Given the hereditary predisposition to tumor formation, the application of certain carcinogens will hasten the appearance and in some instances

9 Basset, J., Wollman, E., Macheboeuf, M. A., and Bardach, M., *C. r. Acad. Sci.*, 1933, **196**, 1138.

10 Basset, J., Lisbonne, M., and Macheboeuf, M. A., *C. r. Acad. Sci.*, 1933, **196**, 1540.

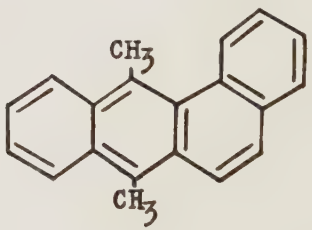
* Finney-Howell Foundation Medical Research Fellowship.

† Summer student from the University of Michigan to the Jackson Memorial Laboratory.

increase the incidence of the tumor. The relationship between carcinogens and the leukemoid conditions has not been so clear-cut. A leukemic condition in mice has been reported following the subcutaneous injection of 1:2:5:6 dibenzanthracene-9:10 *endo-αβ* succinate.[1] Leukemia has been reported in stock mice following painting with 1:2:5:6 dibenzanthracene combined with theelin,[2] and in derivatives of the Bagg albino strain following tar painting.[3] An atypical leukemia has been reported in mice of the S strain following intrasplenic injections of crystalline 3:4 benzpyrene.[4] Recently, a condition of general lymphomatosis has appeared in the dilute brown (dba) strain after the cutaneous application of methylcholanthrene in benzene.[5]

During the course of an experiment testing the carcinogenicity of 9:10 dimethyl-1:2 benzanthracene[6] on different inbred strains of mice

9:10-dimethyl-1:2-benzanthracene.



there appeared in one of the strains used, the dilute brown (dba) strain, individuals with a general lymphomatosis at a strikingly early age. It is the purpose of this report to record these findings.

The dilute brown strain is a high mammary tumor strain with a tumor incidence approximating 85% in the breeding females. Subline 212 is distinct from the other sublines in having a relatively high incidence of lymphoblastomas in old animals. The incidence data are as yet incomplete for this subline.[7] Ten mice of subline 212 were used. On August 1, 1939, when these animals were 4 weeks old, paintings were begun. A 0.3% solution of 9:10 dimethyl-1:2 benzanthracene in thiopene-free benzene was applied to the skin with a

1 Burrows, H., and Cook, J. W., *Am. J. Cancer*, 1936, **30**, 75.

2 Perry, I. H., and Ginzton, L. L., *Am. J. Cancer*, 1937, **29**, 680.

3 Brues, Austin M., and Marble, Beula B., *Am. J. Cancer*, 1939, **37**, 45.

4 Barnes, W. A., and Furth, J., *Am. J. Cancer*, 1937, **30**, 75.

5 Morton, John J., and Mider, G. Burroughs, *Science*, 1938, **87**, 327.

6 Furnished through the courtesy of Dr. W. E. Bachmann of the University of Michigan. See Bachmann, W. E., Kennaway, E. L., and Kenneway, N. M., *Yale J. Biol. and Med.*, 1938, **11**, 97.

7 Personal communication, Dr. G. W. Woolley of this laboratory.

No. 4 camel's hair brush. Paintings were made mid-dorsally from the interscapular to the sacral region with 2 strokes of the brush. These were continued twice weekly at the same site.

Sixty-five days after the first painting ♀$D_2$5 showed extreme bilateral lymphadenopathy of the cervical, axillary and inguinal lymph nodes and had a "swell belly". The animal appeared moribund and was killed. At necropsy there was also lymphadenopathy of the tracheo-bronchial and abdominal-mesenteric nodes. There was enlargement of the liver and spleen. The liver was pale and the spleen grayish in color. There existed a general connective tissue edema. Hydrothorax and ascites were evident. Microscopic examination of the lymph nodes showed an infiltration of lymphoid cells. The lymph node architecture was completely lost and in most cases there was infiltration through the node capsule into surrounding tissue. The nodes were grayish and soft and in most cases were larger than 1 cm in diameter. In some a brownish pigment deposit was found. Liver sinusoids were filled with lymphoid cells and dense areas were formed around the portal vessels. Large areas of the splenic architecture were completely obliterated and pulp tissue was replaced by lymphoblasts.

Five more animals showed a definite general lymphomatosis at 75, 76, 77, 82 and 89 days respectively after the first painting. These animals died from 8 to 15 days after the appearance of lymphadenopathy. In each mouse there was bilateral enlargement of the cervical, axillary or inguinal nodes with accompanying hepatomegaly and splenomegaly. Edema was not so pronounced in these animals and there was little or no hydrothorax or ascites. Histological findings of the lymph nodes, liver and spleen were identical with those described for ♀$D_2$5.

The spleen seemed to be secondarily involved in these animals as there were some regions where the architecture had been preserved. Lymphocyte counts made from blood smears ranged from 87 to 99%. Leukemic infiltration was noted in the mammary gland and connective tissues.

Of the remaining mice, 2 died of infection. ♀$D_2$9 and ♀$D_2$10 showed a moderate lymphadenopathy at 74 and 76 days respectively after the first painting, with an apparent slight subsequent regression. These animals were sacrificed at the age of 133 days. Definite areas of leukemic infiltration were noted in the axillary and inguinal nodes, with a definite capsular infiltration. The liver sinusoids showed the beginning of infiltration, but the architecture of the spleen was normal. These animals probably represent early cases of general lymphomatosis.

Not a single case of lymphomatosis has been observed among the 30 C57 brown mice and 10 A strain mice similarly painted for 145 days, nor among 35 dba, subline 212, control mice.

Summary. A general lymphomatosis has occurred in mice of the dilute brown (dba) strain (subline 212) following painting of the skin with a 0.3% solution of 9:10 dimethyl-1:2 benzanthracene in benzene. Bilateral lymphadenopathy of the axillary, inguinal or cervical lymph nodes appears as early at 95 days after birth, death ensuing within 2 weeks.

11125

On the Free and Combined Silica in Silicotic Lungs.

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While more analytical results for total silica in lungs are needed, there is a much greater need for values for the concentration of free silica, since it is so definitely known to cause silicosis. In the literature on the subject only several publications in the analytical line have to do with free silica in lungs and these reports are limited either to semi-quantitative results[1–4] or to calculations of silica by difference.[5, 6]

By modifying and extending the method of determining free silica in dusts, to lung residues, we have been able to determine minimum free silica. Since the chemical method involves digestion of the finely-divided lung ash residue in hydrofluosilicic acid (H_2SiF_6) in order to dissolve away silicates, and since finely-divided free silica is appreciably soluble in the above acid, we can, however, report only *minimum* free silica values.

The solubility of free silica in hydrofluosilicic acid varies with particle size, among other factors. Particles of silica as long as 10 microns may gain entrance to lungs, although the great majority

1 Sweany, Henry C., Klaas, R., and Clark, G. L., *Radiology*, 1938, **31**, 299.

2 Hicks, Victor, *Instruments*, 1936, **9**, 133; *Ind. Med.*, 1936, **5**, 173.

3 Hicks, Victor, McElroy, O., and Warga, M. E., *J. Ind. Hyg. and Toxic.*, 1937, **19**, 177.

4 Jephcott, C. M., Gray, W. M., and Irwin, Dudley A., *Canadian Med. Assn. J.*, 1938, **38**, 209.

5 Badham, Charles, and Taylor, Harold B., *Med. J. Australia*, 1933, **1**, 511.

6 Jones, William R., *J. Hyg.*, 1933, **33**, 307.

TABLE I.

Silicotic Lung Analyses (Human).

Results are given on the dry lung basis. In each case 5 g of the tissue was taken for treatment with H_2SiF_6.

Lab. No. and case		Ash %	Total silica g	Total silica %	Total silica in ash %	Submersion in H_2SiF_6 Time‡ hr	Submersion in H_2SiF_6 Loss g	Fractions of total silica Free* %	Fractions of total silica Combined† (silicate) %	Age	Autopsy report bearing on silicosis.§
11	T.T.	6.48	.0389	0.389	6.00	342	.3145	83.80	16.19	54	Pneumonoconiosis, pulmonary fibrosis, cardiac dilatation, fibrous nodules in lungs and pulmonary tuberculosis.
15	S.G.	6.57	.1060	1.060	16.26	281	.2371	50.50	49.43	48	Silicosis, pulmonary tuberculosis.
17	M.L.	8.20	.2590	2.590	31.59	174	.3257	50.04	49.96	50	Lungs nodular and solid, pulmonary tuberculosis. Silicosis?
18	L.F.	6.91	.2780	2.780	42.08	377	.3278	10.40	89.56	38	Final stage silicosis.
19	G.H.	7.04	.0830	0.830	11.79	211	.3147	41.50	58.55	55	Severe anthracosis, liver degeneration, not silicosis.
20	B.	11.19	.5360	5.360	48.15	236	.2068	8.28	91.71	67	Dense lung adhesions, pneumonoconiosis, enlarged heart, arteriosclerosis.
22	J.Y.	8.96	.1980	1.980	22.30	395	.4610	8.00	91.91	42	Lungs firm, nodular and fibrotic, anthracotic lymph nodes. Silicosis and tuberculosis.
24	J.LaF.	1.91[1]	.0306	0.306	15.50[3]	92	.0821	41.80	58.17	37	Pneumonoconiosis, healed tuberculosis, right lung abscess.
25	DeM.	3.53[2]	.2650	2.650	75.07[4]	91	.1428	3.25	96.75	52	(Auto accident case.) Hypertrophied and dilated heart.
26	P.R.	5.41	.0430	0.430	7.09	370	.3101	67.00	33.02	43	Pneumonoconiosis and pulmonary fibrosis. "Much silica microscopically." Cardiac hypertrophy and dilatation.
27	C.S.	7.19	.2560	2.560	35.60	298	.3684	11.10	89.06	64	Pulmonary anthraco-silico-tuberculosis.
28	M.DiP.	30.35[5]	.0400	0.400	19.68	93	.0260	91.00	9.00	53	Pneumonoconiosis and pulmonary tuberculosis.
29	J.C.	5.35	.585	0.585	10.95	161	.2620	18.50	81.53	51	Tuberculosis of lower right lung. Diffuse modulation in both. Not typical silicotic nodules.
30	J.W.	7.62	.0910	0.910	11.95	394	.3651	12.30	87.69	49	Clinically chronic endocarditis with aortic stenosis and decompensation. No tuberculosis found. Silicosis?

1,2 This value is probably low because of leaching by the preservative solution.

3,4 This value is probably high because of leaching by the preservative solution

5 This tissue contained an unusually large amount of aluminum, presumably from Portland cement.

* Not corrected for solubility in H_2SiF_6. The values are therefore considered to be minimum.

† These values are considered to be maximum because they are obtained by subtracting minimum free silica from total silica.

‡ Submerged in H_2SiF_6 until loss of weight by dissolving (of silicates) dropped sharply.

§ All cases except Nos. 26 and 30 were known to have been exposed.

found are less than 5 microns, averaging around 1 to 3 microns according to several investigators. Although we have attempted to establish a solubility correction by experiments on fine quartz, so far we have not been successful.

Total silica was determined by the usual gravimetric hydrofluoric acid method. Essentially the free silica method consisted in digesting the ash residues at room temperature with hydrofluosilicic acid and then determining the undissolved free silica by the hydrofluoric acid method.

Table I indicates data and results on 14 silicotic lungs obtained from autopsies in Buffalo and vicinity. More cases would have been desirable but were not available. The table is largely self-explanatory.

The results show that free silica varies greatly both in actual concentration and in percent of total silica; concentrations range from 0.086% to 1.296% of the dry lung; as a part of the total silica the free silica ranges from 3.25% to 91%. The variations are not out of harmony with the fact that different persons are subjected to breathing siliceous dusts of greatly varying compositions in silica and silicates.

It is to be noted that in 5 cases (Nos. 22, 24, 25, 29, 30) the minimum concentration of free silica is within the generally accepted maximum limit of 0.20% of total silica for normal lungs (on the dry basis). The total silica, however, in these cases is well above the normal.

11126

Carbohydrate Metabolism of *Oidium lactis* and *Bacillus subtilis* in Complex Carbohydrate-rich Culture Medium.*

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In previous papers the writer has shown that the rapid growth of pneumococci[1] and streptococci[2] in the usual laboratory media results in a large yield of lactic acid; a small part of the sugar is further

* This study was supported by the Bartlett Memorial Fund and the Douglas Smith Foundation for Medical Research of the University of Chicago. The work was carried out with the assistance of Thaddeus C. Kmieciak.

1 Friedemann, T. E., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 505.

2 Friedemann, T. E., *J. Biol. Chem.*, 1939, **130**, 757.

TABLE I.

Metabolism of Bacteria in Complex Carbohydrate-rich Culture Medium.

Culture medium, meat extract (0.3%), 1% of Bacto- or Witte-peptone, 1 to 1.8% of Na_2HPO_4-12 H_2O, and 0.9% of dextrose. Initial pH 7.6. Temperature 37.5°. Incubated 10 to 24 hours, except *C. diphtheriæ* which was incubated 72 hours.

Organism	Culture medium batch*	Glucose x 2 mm C_3 per l.	Lactic acid mM per l.	Formic acid mM per l.	Acetic acid mM per l.	Ethyl alcohol mM per l.	Undetermined non-volatile acidity cc *N* per l.	Yield of lactic acid %
Eberthella typhosa	B_2	44.9	15.1	26.7	11.0		—3.2	34
Shigella paradysenteriæ (Flexner)	B_2	42.4	14.6	20.5	12.0		0.8	34
Oidium lactis	B_2	52.0	58.8	8.5	3.5		1.0	75
Eberthella typhosa	B_3	51.6	14.7	29.5	17.2	15.2	3.2	29
Oidium lactis	B_3	48.3	34.1	11.4	6.6	5.3	0.9	71
Bacillus subtilis A	B_3	52.3	36.4	10.5	5.9	6.2	0.3	70
" " B	B_3	55.2	38.0	10.5	5.5	6.6	—1.6	69
Corynebacterium diphtheriæ (Park VIII)	B_3	21.8	13.4	3.2	4.0	0	2.1	62
Lactobacillus helveticus (casei ε)	B_3	71.9	64.1	0.3	0.7	1.9	3.6	89

* Date of inoculation: B_2, July 5, 1932; B_3, January, 1939. B_2 contained Bacto-peptone; B_3 contained Witte-peptone.

broken down into 2 moles of formic acid and 1 mole each of acetic acid and ethyl alcohol as in the case of *Eberthella typhosa*.[1, 3] For the first time it was demonstrated that *Escherichia coli* may yield a maximum of 2 moles of formic acid.[1] In the present paper it will be shown that the metabolism of *Oidium lactis* (from milk and dairy products) and *B. subtilis*, the "hay bacillus", both non-pathogenic microörganisms which differ widely morphologically, follows the same pattern.

The bacteriological and analytical methods were the same as described in previous papers.[4] The composition of the culture medium, the temperature, and the duration of incubation are shown at the top of Table I. For the purpose of comparison results are also given from a few other microörganisms which were grown in the same batch of culture medium.

The metabolism of *Eberthella typhosa* and *Shigella paradysenteriae* in culture medium B_2 resulted in a yield of 34% of lactic acid on the basis of the sugar consumed. Slightly less lactic acid was produced by *Eberthella typhosa* in medium B_3. The yield of lactic acid from *Oidium lactis* was high and approximately the same in both media, namely, 75 and 71%. *B. subtilis* yielded 70% of lactic acid in medium B_3, on the basis of the sugar consumed. Another strain of *B. subtilis* gave almost identical yields of products. Both microorganisms converted about one-fifth of the sugar into volatile products which appeared in the medium in approximately the ratio of 2 moles of formic acid to 1 mole each of acetic and ethyl alcohol. Thus in medium B_3, *Oidium lactis* produced 11.4 mM of formic acid, 6.6 mM of acetic acid, and 5.3 mM of ethyl alcohol. *B. subtilis* produced 10.5 mM of formic acid, 5.9 mM of acetic acid, and 6.2 mM of ethyl alcohol. This should be compared with the results from *Eb. typhosa*. From approximately the same quantity of sugar, *Eb. typhosa* yielded 29.5 mM of formic acid, 17.2 mM of acetic acid and 15.2 mM of ethyl alcohol; approximately 60% of the sugar was thus converted into volatile products. Succinic acid was not produced in measurable quantities in either of the media, as measured by the "undetermined non-volatile acidity."

It is evident that these products were produced by anaerobic mechanisms. Under the experimental conditions, even with a long exposure and with slow growth, a high yield of lactic acid probably would have been obtained from these organisms. Such a condition obtained in a culture of *C. diphtheriae*. Although the inoculum was

[3] Harden, A., *J. Chem. Soc.*, 1901, **79**, 610.

[4] Friedemann, T. E., *J. Bact.*, 1938, **35**, 527.

large, growth was very slow and appeared to occur chiefly at the surface. The contents of the bottle were rotated frequently during a period of 72 hours. Despite the obviously aerobic conditions, a yield of 62% of lactic acid was obtained. The yield of volatile products was similar to that obtained in aerated cultures of *Diplococcus pneumoniae*.[1] Neither *Oidium lactis* nor *B. subtilis* produced the maximum of lactic acid from sugar by growth in this medium. The yield of 89% of lactic acid in a culture of *Lactobacillus helveticus* (*casei* ε, v. Freudenreich) perhaps represents the maximum which could be obtained by the growth of microörganisms in this batch of culture medium.

Summary. *Oidium lactis* and *B. subtilis* were inoculated into culture media prepared from 0.3% solution of meat extract or an infusion of beef muscle, to which were added 1% of Witte peptone, 1 to 1.8% of $Na_2HPO_4 \cdot 12\ H_2O$ and 0.9% of dextrose. Both microorganisms grew rapidly at 37.5° and both yielded about 70% of lactic acid on the basis of the sugar consumed. Approximately one-fifth of the C_3-intermediates from the sugar was further metabolized into 2 moles of formic acid and 1 mole each of acetic acid and ethyl alcohol. Their metabolism in the same culture medium is compared with that of *Eberthella typhosa, Shigella paradysenteriae, Lactobacillus helveticus (casei ε)* and *Corynebacterium diphtheriae.*

11127 P

Pantothenic Acid and Nicotinic Acid as Essential Growth Substances for Morgan's Bacillus (*Proteus morganii*).

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Following the announcement by Fildes[1] that nicotinic acid was found to be an essential accessory nutrient for 10 strains of *Proteus,* an investigation was undertaken in this laboratory to determine the nutritional requirements of some 240 strains of *Proteus vulgaris* and other species of the same genus. During the course of the study[2] it was observed that all of the *Proteus morganii* strains exhibited a

1 Fildes, P., *Brit. J. Exp. Path.*, 1938, **19**, 239.

2 Pelczar, M. J., and Porter, J. R., *J. Bact.*, in press.

marked variation from the other *Proteus* cultures. This difference was apparent when the organisms were subcultured in a simple synthetic medium composed of inorganic salts, lactate, and nicotinic acid. None of the Morgan's bacilli was capable of serial subculture in the medium, while practically all of the remaining *Proteus* strains responded with good growth. When this simple medium was supplemented with some 16 amino acids in various combinations the situation was not altered. The absence of some essential growth substance (or substances) appeared to be the likely explanation for the failure of the strains to grow.

This paper is a preliminary report on the nutrition of *Proteus morganii* and the ability of pantothenic acid and nicotinic acid to serve as the essential accessory substances for the growth of the organism.

*Experimental.** Thirty-five strains of *Proteus morganii* constituted the test organisms. Twenty-four-hour cultures on meat infusion agar slants were used as the source of inoculum. The basal medium used throughout was made up of inorganic salts [KH_2PO_4, $(NH_4)_2SO_4$, NH_4Cl, Fe $(NH_4)_2$ $(SO_4)_2 \cdot 6H_2O$, $MgSO_4 \cdot 7H_2O$], glucose, distilled water, and the following amino acids which were incorporated to insure an adequate supply of nitrogenous compounds: dl-alanine, dl-valine, dl-leucine, s-glycine, l-proline, dl-hydroxyproline, dl-aspartic acid, dl-glutamic acid, dl-methionine, dl-phenylalanine, l-tyrosine, d-arginine, l-histidine, dl-lysine, l-tryptophane and l-cystine.

Ten substances which have been identified as growth accessory factors for various organisms were tested alone and in various combinations in the amino acid basal medium. These included: vitamin B_1,† vitamin B_6,† riboflavin,† β-alanine, pimelic acid, nicotinic acid, inositol, cocarboxylase,† glutamine, and cozymase.† Growth of the Morgan's strains failed to occur on subsequent serial transfers in the same medium when one or all of these substances were present in the basic medium.

At this time it was observed that very small amounts of an alcoholic extract of wheat or rice bran stimulated the growth of *Proteus morganii* when added to an otherwise synthetic medium. This led us to believe that panthothenic acid might be an essential growth substance for this organism.

Through the kindness of Professor R. J. Williams, we were able to

* The details of methods and media employed will be reported in a subsequent paper.

† We are indebted to Merck and Company, Inc., for the vitamin B_1 (Betabion), riboflavin, and vitamin B_6, and to Dr. Henry Tauber for the cocarboxylase and cozymase.

test the biological activity of 2 preparations of barium pantothenate, one labeled as 20% pure and the other as 88% pure. When either of these 2 pantothenate preparations was added in quantities of one μg to 10 ml of the basal amino acid medium plus all 10 of the above growth substances, a luxuriant growth occurred within 24 hours after inoculation and continued through 5 subsequent transfers on a similar medium. Further experiments showed that the pantothenate preparation alone would not support growth. Consequently several media were prepared using various combinations of the 10 previously mentioned growth factors along with the pantothenate. By a process of elimination it was observed that when nicotinic acid and pantothenate or cozymase and pantothenate were present in the basal medium, the resulting growth was good. Pantothenate in combination with the other growth substances gave negative results. It is not surprising that cozymase could be substituted for nicotinic acid since it is generally accepted that the nicotinic acid molecule is used in the synthesis of cozymase. A quantitative comparison of the biological activity of the pantothenate preparations is given in Table I.

Although neither sample is pure, we are informed[3] that the impurities present are physiologically inactive. It will be observed that the activity of the 88% pure preparation of barium pantothenate (II) is roughly 4 times that of the 20% preparation (I). The former was easily detectable at 0.01 μg per 10 ml of medium. This fact together with the effectiveness of the material in such an infinitesimal concentration seems to be sufficient evidence for pantothenate to be classed as a growth factor for *Proteus morganii.*

To our knowledge, this is the fourth instance in which pantothenic

TABLE I.

Quantitative Comparison of Biological Activity of 20% and 88% Pure Barium Pantothenate with *Proteus morganii* as Test Organism.

20% barium pantothenate (I)		88% barium pantothenate (II)	
μg added per 10 ml of medium*	Visible growth	μg added per 10 ml of medium	Visible growth
.0	0	.0	0
.01	0	.0025	0
.04	++	.01	+
.1	++	.025	++
.4	+++	.10	+++
1.0	+++	.25	+++

+++, good growth; ++, moderate growth; +, poor growth.

* Medium: Nicotinic acid, 16 amino acids, inorganic salts, glucose and distilled water.

3 Williams, R. J., personal communication.

acid has been demonstrated to serve as a growth factor for certain bacteria. It has previously been reported as an essential growth factor for *Corynebacterium diphtheriae* (Mueller and Klotz,[4] Evans, Handley and Happold[5]), for the lactic and propionic acid bacteria (Snell, Strong and Peterson[6]) and for the hemolytic streptococcus (Subbarow and Rane,[7] and McIlwain[8]).

Summary. Evidence has been presented which indicates that pantothenic acid and nicotinic acid are the essential growth factors for *Proteus morganii.*

11128

Urea-Treated Virus as a Vaccine against Rabies.*

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Urea and allied compounds have been studied with regard to their denaturing action on proteins[1, 2] and their inactivating effect on viruses.[3, 4] MacKay and Schroeder mixed rabies-fixed virus and poliomyelitis virus with concentrated urea solutions; complete inactivation of these viruses and loss of antigenicity resulted. The work here reported concerns the action of urea solutions on rabies-fixed virus.

In the first experiment fresh rabbit-fixed virus was ground with a saturated (room temperature) solution of urea in normal saline to make a 50% suspension of tissue. The resulting mixture was smooth

4 Mueller, J. H., and Klotz, A. W., *J. Am. Chem. Soc.*, 1938, **60**, 3086.

5 Evans, W. C., Handley, W. R. C., and Happold, F. C., *Brit. J. Exp. Path.*, 1939, **20**, 396.

6 Snell, E. E., Strong, F. M., and Peterson, W. H., *J. Am. Chem. Soc.*, 1938, **60**, 2825; *J. Bact.*, 1939, **38**, 293.

7 Subbarow, Y., and Rane, L., *J. Am. Chem. Soc.*, 1939, **61**, 1616.

8 McIlwain, H., *Brit. J. Exp. Path.*, 1939, **20**, 330.

* This research was supported by a grant from the W. J. Matheson Fund for the study of encephalitis and by the Rabies Research Fund of the University of Southern California.

1 Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, 1929, **13**, 121.

2 Greenstein, J. P., *J. Biol. Chem.*, 1938, **125**, 501.

3 Stanley, W. M., and Lauffer, M. A., *Science*, 1939, **89**, 345.

4 MacKay, E. M., and Schroeder, C. R., PROC. SOC. EXP. BIOL. AND MED., 1936, **35**, 74.

and creamy and flowed easily. Numerous bubbles of fat floated to the surface and were removed. The remaining material was homogeneous (save for small fragments of fibrous tissue, bits of blood clot and insoluble particles of a fatty consistency, all of which were removable by straining through gauze) and could be drawn through a 27 gauge needle. In 4 subsequent experiments less urea was utilized; these vaccines (Nos. 2, 3, 4 and 5) contained 40% rabbit-fixed virus brain and 60% of ⅓ saturated urea in normal saline. All vaccines, after preparation, were incubated at 37°C for 24 or 48 hours and were subsequently preserved at refrigerator temperature.

The undiluted vaccines of the first 2 experiments proved extremely irritating and rapidly killed a number of mice, when injected intracerebrally in doses of 0.02 cc to 0.03 cc in order to test for the presence of active virus. Three mice, which survived the initial cerebral irritation resulting from these test injections and which thereafter remained normal, indicated that the first vaccine was non-infectious for mice under the experimental conditions employed. The second vaccine killed all test mice shortly after intracerebral inoculation; its active virus content was, therefore, not determined. The last 3 vaccines were diluted 5 times with normal saline, before intracerebral inoculation, in order to minimize their direct irritating effects and injection of these vaccines produced no immediate untoward symptoms. The third and fifth vaccines contained active virus and produced rabies in a number of these test mice. Vaccine No. 4, however, was non-infective for 8 test animals, which were observed for a period of 18 days and which were subsequently shown to be fully susceptible to intracerebral infection with small doses of rabies virus. It was, therefore, concluded that vaccine No. 4 contained either no active virus or that its concentration of infective material was reduced to a minimum.

All 5 vaccines were tested for their immunizing efficacy. Vaccine No. 1 (which contained a high concentration of urea) was administered intraäbdominally in 8 daily doses, each of 0.025 cc, to 14 white Swiss mice. An intracerebral titration with fixed virus performed on these mice 2 weeks after active immunization was begun and compared with adequate untreated controls, indicated that no demonstrable immunity had developed. The last 4 vaccines, which contained less than half the amount of urea that was present in vaccine No. 1, showed very definite immunizing properties, however, when injected in like manner. Vaccines Nos. 3, 4 and 5 were injected in 2 different ways: (a) in 8 daily intraperitoneal doses of 0.025 cc, (b) in 1 dose of 0.20 cc. Controlled intracerebral titrations for immunity were

performed as above. The samples of rabbit brain-fixed virus, employed in these titrations, were removed from 50% glycerine in the first 4 experiments, while fresh virus was employed in test No. 5. Virus was ground to an initial 1/5 suspension in the first 3 tests, whereas a 1/10 suspension was prepared for the last 2 tests. These initial preparations were centrifuged at a moderate speed for a few minutes and the supernatants were further diluted by tens. Intracerebral test doses consisted of 0.02 cc to 0.03 cc of these dilutions. Normal saline was used as a diluent in the first 2 experiments and distilled water was employed after this. All mice were observed for a minimum of 14 days before being classified as "survivors".

Table I summarizes experiments on the immunizing powers of the last 4 vaccines, all of which tests yielded comparable results.

The table indicates that 8 intraäbdominal injections of urea-treated vaccines (Nos. 2, 3, 4, and 5) containing 40% rabbit-fixed virus brain and 60% ⅓ saturated urea in saline, produced a high degree of immunity against 1 and 10 minimal infective doses of rabies-fixed virus and some immunity against even greater doses. In addition, 3 of these vaccines (Nos. 3, 4 and 5) when given in like total amounts but in one large dose, produced an immunity of a somewhat lower grade.

Summary. Rabbit brain rabies-fixed virus exhibited considerable resistance to the action of urea. This virus maintained its antigenicity in 4 experiments and its infectivity in, at least, 2 tests in the presence of a concentration of urea which was sufficiently high to liquefy most of the virus-containing brain material.

TABLE I.
Resistance of Mice Immunized with Urea Vaccine.
All vaccinated mice received the same total amount of vaccine.

Intracerebral test virus M.I.D.'s	Vaccine given in: Eight injections		One injection		Untreated controls	
	Survivors	Deaths	Survivors	Deaths	Survivors	Deaths
10,000	2	3	0	4	0	4
1,000	0	4	0	4	0	4
100	14	6	5	7	0	10
10	15	1	6	6	0	24
1	18	0	11	5	1	24

11129

Attempt to Modify Growth, Development and Tumor Incidence in Mice with Thymus Gland Extracts.

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Spontaneous mammary cancer in mice is predominantly a disease of mature or old females and is influenced in many strains by their breeding activity. It, therefore, becomes of great interest to modify reproductive activity, development and growth in strains of mice of known rates of cancer susceptibility to determine the relationship between the chronological and physiological age of tissues and the appearance of cancer.

The biologic effects of thymus gland extracts on rats reported by Rowntree, Clark and Hanson[1] and Rowntree[2] were precisely of the nature we desired to produce in mice. These were: increase in number and size of litters and birth weight of offspring; and, in the third generation of treated rats, precocious development accompanied by early eruption of teeth, appearance of fur, opening of eyes, descent of the testes or opening of the vagina and earlier sexual maturity.

The present experiments were undertaken with the Line A strain of albino mice, in which the tumor incidence shows a high correlation with breeding activity. Approximately 5% of virgin females develop tumors at 11 months of age. In bred females, which develop tumors at an average age of 15 months, the percentage of tumors increases with the number of litters borne: 12.56% in those with 1; 24.44% in those with 2; and 34.21% in those with 3 litters.

Three possible effects of thymus gland extract injections presented themselves. First, with an increase in the number of litters, a rise in tumor incidence might be anticipated; on the other hand, if the extract produced maturation and differentiation of cells, such an effect might either prevent the appearance of tumors, which are typically of undifferentiated cells, or, by bringing about precocious physiological maturity, hasten the onset and increase the number of tumors.

Extracts of thymus glands from young calves were prepared in this laboratory by the method then employed by Rowntree (personal communications, Dec., 1935) and their potency in terms of reduced

1 Rowntree, L. G., Clark, J. H., and Hanson, A. M., *J. A. M. A.*, 1934, **103**, 1425.
2 Rowntree, L. G., *J. A. M. A.*, 1935, **105**, 592.

TABLE I.
Females Receiving Thymus Gland Extract in F_1, F_2, F_3 Generations from Treated Parents.

Generation	No. ♀s bred	Died before 3 mo. without offspring	Survived after 3 mo.	Fertile					Sterile	
				No.	Range in age at death, mo.	No. of litters	No. of offspring ♂	No. of offspring ♀	No.	Range in age at death, mo.
F_1	7	2	5	4	8-15	7	23	15	1	
F_2	14	6	8	2	3- 6	2	6	3	6	1-24
F_3	3	0	3	0		0			3	14-22
Totals	24	8	16	6		9	29	18	10	

glutathione content determined. Extracts containing from 28 mg % to 110 mg % reduced glutathione were administered by intraperitoneal injections of 0.5 cc 3 times a week from weaning throughout the reproductive period. They were stored in small sterile vials in the refrigerator. An opened vial was never used for more than 3 weeks and no extracts were more than 12 weeks old when injected. The material was found to be toxic if given at more frequent intervals.

After a number of preliminary experiments, a litter of 3 females and 1 male was selected to start a line, all females and males (*i. e.*, those used for breeding) of which received thymus gland extract in each generation. Of the 3 original females, 1 died at 4 months, with no offspring, 1 at 6 months, having had 1 litter of 4, and the third at 21 months, having had 3 litters totalling 15 young. This mouse developed a tumor at 20 months of age. The progeny in succeeding generations are listed in Table I. It will be seen that contrary to the expected increase in number and size of litters, the fertility in the second generation decreased markedly, and only 3 third-generation females were born. These 3 females were sterile.

Birth weights and growth increments of young in the F_1, F_2 and F_3 generations were well within the normal range as compared with the controls. There was no evidence of developmental precocity.

Mammary gland tumors appeared at 20 months in the F_0 female which had had 3 litters and at 24 months in 1 sterile F_2 female. Leukemia developed at 14 months in 1 sterile F_3 female. Thymus gland extract, therefore, did not inhibit the onset of spontaneous tumors in mice which had received it over a considerable part of their life span. Because of decreased fertility, the number of animals available for the observation of tumor incidence was too small to permit drawing any other conclusions. Nineteen females of the experimental group survived to tumor age and only 3 of these had been fertile. Injections of extract into 1 female with spontaneous mammary cancer had no noticeable effect on the tumor.

From the findings of Rowntree, *et al.*, the potency of thymus gland extract appeared (at the time these experiments were started) to be linked with its sulfhydryl content. Based upon this consideration, a parallel experiment was carried out with *p*-thiocresol.* This choice was determined by the reports of Reimann[3] and Reimann and Ham-

* The *p*-thiocresol was obtained from the Eastman Kodak Company and made up as described by Reimann;[2] *i. e.*, 0.01 g dissolved in 5 cc of 95% ethyl alcohol, to which was added 100 cc distilled water, a dilution of approximately 1:10,000. Intraperitoneal injections of 0.5 cc of this solution were given 3 times weekly.

[3] Reimann, Stanley P., *J. A. M. A.*, 1930, **94**, 1369.

mett[4] concerning its stimulating effect on wound healing and cell proliferation, and by the work of Hammett[5, 6] on the action of the SH-radical in stimulating mitosis.

In this experiment ρ-thiocresol was injected into 5 generations of Line A albino mice from weaning at 21 days of age throughout their breeding period. The material was not toxic and the mice withstood the injections well. No change in fertility, growth or development ensued and there was no significant difference in cancer incidence in the 5 generations of treated animals or between treated mice and stock Line A females of similar breeding history.

Injections of ρ-thiocresol were likewise given to 10 mice with inoculated tumors (tumor 478, an adenocarcinoma which grows progressively on inoculation into Line A strain mice) to test the effect of the material on the growth of tumor cells. Treatment was begun while the tumors were still small. Daily injections of 0.5 cc ρ-thiocresol in 1:10,000 dilution were given intraperitoneally to 5 and subcutaneously to 5. After 1 week, during which the tumors had grown at their normal rate, injections of ρ-thiocresol in dilution of 1:5000 were given in the same manner but on alternate days for a period of 10 days. All tumors grew steadily and there was no evidence of either stimulation or inhibition.

Summary. Thymus gland extract was without stimulating effect on growth or development in Line A albino mice when injected in doses of 0.5 cc 3 times weekly from the time of weaning throughout the reproductive period. A marked lowering of fertility occurred in the second generation from treated parents and grandparents. There were only 3 third-generation females and these were sterile. This unexpected decrease in fertility resulted in too small a number of females surviving to tumor age to permit any conclusions as to the effect of thymus extract upon tumor incidence other than that it did not prevent their appearance or affect their growth.

ρ-Thiocresol was without effect on fertility, growth or development of Line A albino mice and did not modify the occurrence of spontaneous tumors. It did not influence the growth of inoculated tumors.

[4] Reimann, Stanley P., and Hammett, Frederick S., PROC. SOC. EXP. BIOL. AND MED., 1929, **27**, 20.

[5] Hammett, Frederick S., *Protoplasma*, 1931, **13**, 331.

[6] Hammett, Frederick S., *Arch. Path.*, 1929, **8**, 575.

11130

Spectacled Eye Condition in Rats.*

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In a previous paper we reported the occurrence of a specific deficiency syndrome in rats which was termed "spectacled eye condition".[1] This condition was differentiated from acrodynia, paralysis and hemorrhagic disease. The eye erosions were cured by the fullers earth filtrate of liver extract but not by the eluate or crystalline vitamin B_6. Corn oil appeared to interfere with the production of the spectacled eye symptoms. In this paper we wish to report further studies on this condition.

The ration used previously (ration J_{29}) had the following composition: sucrose 78%, purified casein 18%, and salts IV 4%, supplemented with 1.2 mg thiamin, 2.0 mg riboflavin, 300 mg choline and 300 mg nicotinic acid per kilo of diet. Two drops of haliver oil were given each rat per week.† The treatment of the animals and preparation of the diet were as previously described.[1] This ration was not entirely satisfactory for these studies as many rats failed to survive long enough to develop the specific symptoms desired. Consequently we sought a suitable supplement which would afford survival and a certain amount of growth and yet permit the development of severe deficiency symptoms.

Two materials were tested at several levels, a dehydrated cereal grass‡ and a liver extract prepared by Dr. Klein.§ The results are shown in Table I and include the average weights at 5 weeks and the deficiency symptoms observed. These symptoms are identical with those already described in detail.[1] In addition to the symptoms listed

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1 Oleson, J. J., Bird, H. R., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1939, **127**, 23.

† The thiamin, nicotinic acid and vitamin B_6 were supplied by Merck and Company, haliver oil by Abbott Laboratories and the alpha-tocopherol by Hoffman-LaRoche Company.

‡ This is a dehydrated cereal grass furnished by the Cerophyl Laboratories, Kansas City, Missouri.

§ This liver fraction (No. 36872) was furnished by Dr. David Klein, Wilson Laboratories, Chicago, Illinois.

TABLE I.
Effect of Liver Extract and Grass on Growth and Symptoms.

Ration	Supplement	No. of rats	Initial wt, g	Wt at 5 weeks, g	Symptoms
J_{29}	1% L.E. 36872	2	41	71	Dermatitis, spectacled eye, paralysis, scaly ears, hemorrhagic disease.
J_{29}	2% L.E. 36872	2	41	104	Dermatitis, sl. spectacled eye
J_{29}	4% L.E. 36872	2	40	124	No symptoms
J_{29}	1% grass 1109-5B	2	37	46	Dermatitis, paralysis
J_{29}	2% " 1109-5B	2	43	57	Dermatitis, paralysis, 1 dead
J_{29}	4% " 1109-5B	2	37	57	1 sl. dermatitis 1 spectacled eye
J_{29}	6% " 1109-5B	2	36	64	No symptoms
J_{29}	8% " 1109-5B	2	38	59	" "

in the table, all of the rats showed severe scaly tails and paws, typical of linoleic acid deficiency and also the testicle atrophy in males and delayed vaginal openings in females reported by McKibbin, *et al.*[2] The rats receiving the liver extract grew considerably better than those on the grass, which evidently lacks certain factors present in liver. Since the rats receiving ration J_{29} plus 1% of the liver extract showed severe symptoms, this level was used for all of the assays reported in this paper.

Rats receiving ration J_{29} plus 1% liver extract developed acrodynia and the spectacled eye condition in about 6 weeks. The addition of 10 gamma of crystalline vitamin B_6 per rat per day cured the acrodynia in about 3 weeks but the eye erosions remained unchanged. This further demonstrates the inactivity of vitamin B_6 in curing the spectacled eye syndrome. If vitamin B_6 crystals were fed from the beginning, the acrodynia was prevented and the onset of the eye condition was delayed somewhat, due to the increased food consumption of the rats, but when the condition developed it was as severe as in the other cases.

Of the materials tested for their ability to cure the spectacled eye condition, corn oil proved to be the most active. Two or 3 drops of corn oil per day would heal severe eye erosions in 3 weeks. This supplement would also cure the scaly tails and cause the testes of the rats to return to normal. Alpha tocopherol was completely inactive at 50 gamma per week with respect to the eye condition or testes.[2]

The fuller's earth filtrate of liver extract was again found to be active, although the cures were slower than in the case of corn oil.

2 McKibbin, J. M., Oleson, J. J., Elvehjem, C. A., and Hart, E. B., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 25.

Healing took place in 4 to 6 weeks when the filtrate, made by our usual method,[1] was fed equivalent to 250 mg liver extract per rat per day.

A purified concentrate of pantothenic acid was fed at a level furnishing 100 gamma of pantothenic acid per rat per day. This concentrate, treated with alkali to completely inactivate the pantothenic acid, was also fed at the same level. The preparation of these fractions was described by Oleson, *et al.*,[3] and their pantothenic acid content determined by bacterial assay.[4] Both of these supplements showed equal activity, but appreciably less potency than the liver filtrate. Healing took place in about 8 weeks. This definitely eliminates pantothenic acid as the factor concerned. The results are given in Table II.

From the evidence presented in this and our earlier work, we conclude that there is present in liver extract a factor which prevents the characteristic erosions about the eyes in rats. Its relation to other factors which we have studied, namely pantothenic acid,[3] factor W,[5] and the factor preventing nutritional achromotrichia,[6] can be at least indicated. The slow activity of the pantothenic acid concentrate and the apparent stability of the spectacled eye factor to alkali eliminates this vitamin. In our earlier work the spectacled eye condition was cured by filtrates having very little factor W potency,[1] which at least indicates that factor W is not concerned. The inability of corn oil to prevent nutritional achromotrichia (greying of the hair) is indirect evidence that these 2 factors are not identical. Whether this factor is identical with the so-called "accessory factor" in the filtrate, required in addition to vitamin B_6 for the complete healing of acrodynia

TABLE II.
"Spectacled eye" Preventing Potency of Supplements.

No. of rats	Ration	Supplement	Avg healing time, wk
5	J_{29} + B_6 + 1% liver extract	Corn oil	3
4	J_{29} + B_6 +1% liver extract	Alpha tocopherol	Inactive
6	J_{29} + B_6 + 1% liver extract	Fuller's earth filtrate	4-6
2	J_{29} + B_6 + 1% liver extract	Pantothenic acid concentrate	6-8
2	J_{29} + B_6 + 1% liver extract	Alkali inactivated pantothenic acid concentrate	6-8

3 Oleson, J. J., Woolley, D. W., and Elvehjem, C. A., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 151.

4 Snell, E. E., Strong, F. M., and Peterson, W. H., *Biochem. J.*, 1937, **31**, 1789.

5 Elvehjem, C. A., Koehn, C. J., and Oleson, J. J., *J. Biol. Chem.*, 1936, **115**, 707.

6 Oleson, J. J., Elvehjem, C. A., and Hart, E. B., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 283.

is not certain as yet, but the action of corn oil suggests a possible relationship.[7, 8, 9] It is to be emphasized, however, that we observe these eye lesions in the complete absence of the symptoms of acrodynia. The existence of eye erosions distinct from acrodynia has been recently reported by György and Eckardt,[10] but whether their "type III" dermatitis is the same as the spectacled eye condition remains to be shown. The solution of the acrodynia problem may give the solution to the "spectacled eye" problem.

The fact that the scaly tail condition also appears in the rats which show the "spectacled eye" condition and the fact that corn oil cures both conditions suggests a possible relationship between these two conditions. However, it should be pointed out that liver extract and liver extract fractions cure the spectacle eye condition without producing any effect on the scaly tail condition. It should also be pointed out that McKibbin, *et al.*,[2] have produced the scaly tail condition on diets containing 4% liver extract which supplies ample amounts of the "spectacled eye" factor.

Summary. The curative action of corn oil in healing the "spectacled eye" condition has been demonstrated. The identity of the factor in liver which prevents these symptoms with the other members of the vitamin B complex has been discussed. Direct and indirect evidence is offered that, besides the crystalline vitamins contained in the ration, the factor concerned is not identical with pantothenic acid, factor W and the factor preventing nutritional achromotrichia. A possible relationship of this factor to the so-called "accessory factor" preventing acrodynia and of this condition to the "type III" dermatitis of György and Eckardt is pointed out. Further, the relation of this condition to the deficiency of the essential fatty acids is discussed.

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7 Birch, T. W., *J. Biol. Chem.*, 1938, **124**, 775.

8 György, P., *J. Am. Chem. Soc.*, 1938, **60**, 983.

9 Kuhn, R., and Wendt, G., *Ber. Deutsch. Chem. Gesell.*, 1938, **71**, 780.

10 György, P., and Eckardt, R., *Nature*, 1939, **144**, 512.

11131

Plasma Electrolytes as Affected by Insulin and by Varying Partial Pressures of Atmospheric Gases.

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In recent studies on the mechanism of insulin convulsions in which the decreases in plasma potassium and inorganic phosphorus, as well as the degree of hypoglycemia, were measured at regular intervals, it was found that the level of blood sugar alone bears a constant relationship to the convulsion.[1] Further experiments on the effects of varying partial pressures of atmospheric gases, O_2, N_2 and CO_2, showed that a drastic lowering of the O_2 content or a marked increase in the CO_2 of the respired air tended to prevent insulin convulsions entirely or greatly to delay their onset in the normal fasting dogs, even when the blood sugar was depressed to levels as low as or lower than those previously observed in the same animals before convulsions.[2] The latter finding indicates that some other factor in addition to hypoglycemia plays a rôle in the mechanism of insulin convulsions.

In the hope of obtaining further information on this phase of the problem, the present studies pertaining to changes in the plasma electrolyte patterns under the foregoing conditions were undertaken. The procedure followed was that of determining the various electrolytes of the plasma before and at regular intervals after administration of convulsive doses of insulin to fasting unanesthetized dogs, first in room air and subsequently in the various abnormal atmospheres referred to above. No 2 experiments on the same animal were carried out at intervals shorter than one week. The plasma electrolyte patterns were determined as follows: 12 times in 5 normal fasting dogs in room air; 3 times in 3 of the same dogs between 210 and 240 minutes after administering convulsive doses of insulin but before convulsions occurred; twice in 2 of the dogs 15 minutes following the insulin convulsion; 3 times in 3 of these animals after 150 minutes in a tent containing approximately 5% O_2 and 95% CO_2; twice under the latter conditions on 2 of the same dogs but with the administration of previously determined convulsive doses of insulin; twice in 2 dogs after 150 minutes in an atmosphere containing

1 Ziegler, Mildred R., and McQuarrie, Irvine, PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 142.

2 McQuarrie, Irvine, and Ziegler, Mildred R., PROC. SOC. EXP. BIOL. AND MED., 1938, **39**, 525.

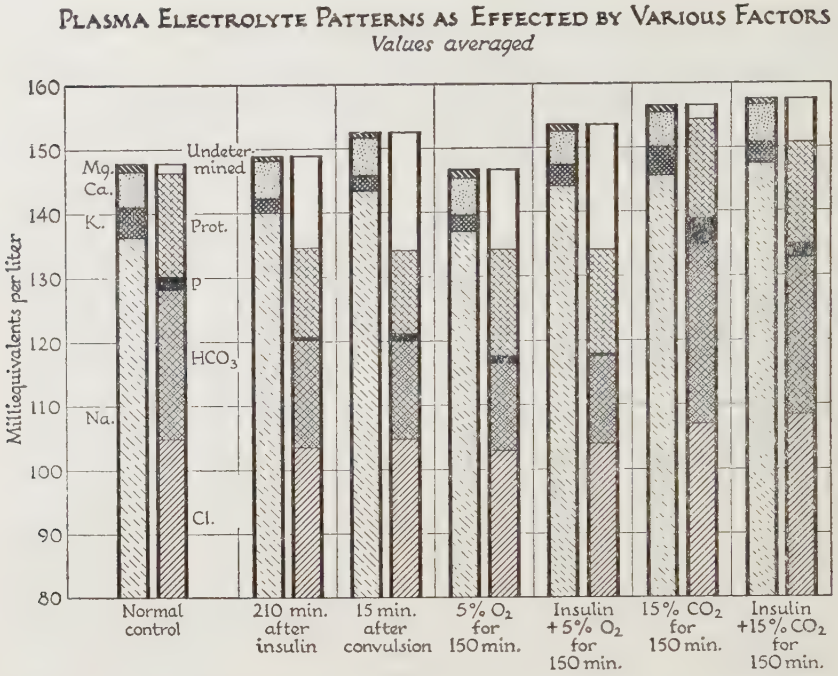

FIG. 1.

Plasma electrolyte patterns as affected by breathing atmospheres of various concentrations of O_2 and CO_2 with and without large doses of insulin.

approximately 15% CO_2, 20% O_2 and 65% N_2 and twice in the latter animals under the same conditions but with the administration of large doses of insulin. Convulsions did not occur during any of the experiments in which the animal was made to breathe the low O_2 or high CO_2 atmospheres.

For the sake of brevity and clearness the results are presented in graphic form in Fig. 1. Since the data from similar experiments were found to be consistent, average values obtained under the various conditions involved were employed in construction of the figure. Comparison of the different columns in the figure, which represent total values for the individual bases and acids of the plasma, shows the following changes, due to the various experimental conditions. Comparing the data for each of the experiments with those shown for the composite normal control shows that insulin in convulsive doses causes a fairly marked decrease in potassium and inorganic phosphorus and moderate decreases in plasma carbon dioxide content and protein. The sodium, calcium, magnesium and chloride remained practically unchanged, whereas the undetermined acid fraction is moderately increased. Fifteen minutes after the insulin con-

vulsion the inorganic phosphorus was found to have risen somewhat, the sodium was slightly increased over the preconvulsive level and the undetermined acid fraction increased still more. The most striking effect of breathing an atmosphere containing 5% O_2 was a fairly marked decrease in potassium, phosphorus and carbon dioxide content. The sodium, calcium, magnesium and protein were quite unaffected. The undetermined acid fraction was considerably increased. When insulin was given in large doses while the animals were left in the atmosphere containing 5% O_2 the sodium was increased by 8 milliequivalents per liter while both carbon dioxide content and the inorganic phosphorus were greatly decreased. The potassium showed a moderate decrease, also. The undetermined acid fraction was greatly increased above normal. The electrolyte pattern for this latter type of experiment in which the animal did not have a convulsion shows a striking resemblance to that found shortly after an insulin convulsion.

In contrast with the effects of anoxic anoxia the breathing of 15% CO_2 resulted in a slight increase in sodium, practically no change in potassium, calcium, magnesium, chloride and protein, but a significant increase in carbon dioxide content and a very marked rise in the inorganic phosphorus. The undetermined acid fraction remained essentially normal. Administration of insulin in large doses during the period of high CO_2 breathing caused but a slight increase in the undetermined acid fraction. The potassium and magnesium were moderately decreased. The sodium and phosphorus were moderately increased while the calcium, chloride, protein and carbon dioxide content remained unchanged.

Breathing an atmosphere containing approximately 5% O_2 and 95% N_2 or one containing 15% CO_2, 20% O_2 and 65% N_2 caused hyperglycemia in the normal animal. After adrenalectomy fairly marked hypoglycemia resulted from breathing the 5% O_2 atmosphere while slight hyperglycemia still resulted from breathing 15% CO_2. The effects of breathing an atmosphere containing but 5% O_2 on the plasma electrolyte pattern were found to be essentially the same in the adrenalectomized as in the normal animal.

It may be concluded that the results of this study do not indicate the existence of any definite relationship between changes in the electrolyte pattern and the occurrence of convulsions due to insulin.

11132 P

Influence of Specific Serum Therapy on Plasma Lipids in Pneumonia.*

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The author has thoroughly studied the changes which occur in the plasma lipids during acute infections in children. The investigations have included pneumonia.[1, 2] The patients did not receive any specific treatment during the febrile period of the disease. It has been observed that there is a definite lowering of the values for total cholesterol, total fatty acids, and phospholipids during the height of the infection as compared with the levels during convalescence. The drop in the total cholesterol is due to a marked fall in the ester cholesterol content of the blood. The low total fatty acid values are accompanied by a definite reduction in the iodine absorption values resulting in a low iodine number for the serum fatty acids. The decrease in the phospholipid content of the serum is accompanied by a rise in the iodine absorption values for the phospholipid fatty acids. This rise is, however, quickly followed by a marked fall so that toward the end of the height of the illness very low iodine numbers are obtained for the fatty acids of the phospholipids.

The lipids have been closely followed during the convalescent period. It has been noted that the cholesterol values rise first to normal and in some instances go above the normal range for a short period of time. The total fatty acids increase slowly during the first phase and more rapidly to normal during the last part of convalescence. The phospholipids with the low iodine number of their fatty acids rise very slowly and do not reach the normal range until the patient has fully recovered and there is no residual infection.

These observations in untreated patients during the natural course of the pneumonia prompted an investigation of the influence of serum therapy on the lipid changes. Three children were carefully selected for the preliminary study which is presented in this paper. The subjects were 6, 7, and 8 years of age, all ill with lobar pneumonia due to the pneumococcus, Type I. They received the same dose of pneumococcus Type I serum—namely 40,000 units. It

* This work was supported by a grant from the Medical Research Fund of the University of Minnesota and from the Mead Johnson and Company.

1 Stoesser, A. V., and McQuarrie, Irvine, *Am. J. Dis. Child.*, 1935, **49**, 658.

2 Stoesser, A. V., *Am. J. Dis. Child.*, 1938, **56**, 1215.

was administered intravenously soon after admission of the child to the hospital. During the period of observation little medication was given to the patients. The administration of fluid or blood subcutaneously, intramuscularly, or intravenously was not permitted. The first sample of blood was obtained just before the serum was given. At this time each child had only been ill from 24 to 48 hours and the temperature ranged between 40° to 40.5°C. The second sample was collected about 24 hours after the temperature had fallen to normal. The third and fourth blood samples were drawn on the fourth and seventh days of the afebrile convalescent period respectively. Each time the blood sample was collected, the patient was thoroughly examined and a roentgenogram of the lungs was obtained. Bloor's methods[3, 4, 5] were used to determine the total, ester and free cholesterol values. The microgravimetric technic of Wilson and Hansen[6, 7] was employed in studying the other serum lipids. The Rosenmund-Kuhnhenn method as described by Yasuda[8] was used to determine the iodine absorption of the serum fatty acids.

The results are summarized in Table I.

TABLE I.

Plasma Lipids of Pneumonia Before and After Administration of Specific Serum.

Case No.	Total cholesterol				Cholesterol esters				Free cholesterol			
	Mg per 100 cc serum											
	A	B	C	D	A	B	C	D	A	B	C	D
1. P.H.	122	225	234	221	70	146	152	162	52	79	82	59
2. R.S.	117	157	240	219	72	89	158	152	45	68	82	67
3. D.C.	130	242	243	250	75	156	183	195	55	86	60	55
Avg	123	208	239	230	72	130	164	169	50	77	74	60

	Total fatty acids				Iodine number				Phospholipids				Iodine number*			
	Mg per 100 cc serum															
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
1. P.H.	289	386	452	440	96	101	107	114	113	146	152	150	134	114	112	121
2. R.S.	296	546	401	456	98	90	101	93	99	126	116	126	109	113	107	103
3. D.C.	297	423	463	378	91	98	96	100	87	115	110	173	107	113	123	115
Avg	294	451	438	424	95	96	104	102	99	129	126	149	116	113	114	113

* Iodine number of the phospholipid fatty acids.
A—Blood sample collected before serum administered.
B—Blood sample collected 24 hours after temperature normal.
C—Blood sample collected on 4th day of convalescence.
D—Blood sample collected on 7th day of convalescence.

3 Bloor, W. R., *J. Biol. Chem.*, 1916, **24**, 227.

4 Bloor, W. R., and Knudson, Arthur, *J. Biol. Chem.*, 1916, **27**, 107.

5 Bloor, W. R., personal communication to the author.

6 Wilson, W. R., and Hansen, A. E., *J. Biol. Chem.*, 1936, **112**, 457.

7 Hansen, A. E., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 376.

8 Yasuda, M., *J. Biol. Chem.*, 1931-32, **94**, 401.

The similarity of the infections in the 3 subjects makes the results of the study most interesting. The cholesterol values were already subnormal before the specific serum was administered. The response to the single dose of serum was a drop in the temperature in each instance to normal within 12 to 24 hours, and one day later when the second blood sample was collected the total cholesterol and esters had already returned to the normal range. In case 2 the rise was not rapid and in this child the roentgenogram revealed a slight increase in the extent of the pneumonic process in the lung. However, after the fourth day of convalescence, all cholesterol values were normal and the roentgenograms showed complete resolution of the pneumonia.

The total fatty acids and the phospholipids were slightly depressed before the administration of serum. Immediately following the fall in temperature, there was a rapid increase in the total fatty acids while the phospholipids rose more slowly. The iodine numbers of the total fatty acids and of the phospholipid fatty acids underwent very little change for the prompt response to serum therapy apparently prevented any marked drop. The intensity of the fat metabolism during pneumonia and the presence of bacterial toxins may be responsible for the fall in the plasma lipids to abnormal levels. The early use of a specific serum tends to control this altered lipid metabolism which is present in acute infections such as pneumonia.

11133

Lymph Flow from the Heart-Lung Preparation During Pulmonary Edema.

VICTOR LORBER. (Introduced by M. B. Visscher.)

From the Department of Physiology, University of Minnesota.

The absence of demonstrable lymphatics beyond the alveolar ducts[1] suggests a factor in the rapid development frequently characterizing pulmonary edema. In the following experiments this anatomical fact was put to a functional test.

In 4 dogs under nembutal anesthesia the thoracic duct was cannulated one centimeter from the point of its entrance into the left subclavian vein. Following this, a Starling-Knowleton heart-lung preparation was made, and the thoracic duct tied off above the dia-

[1] Maximow, A. A., and Bloom, W., *A Textbook of Histology*, W. B. Saunders Co., 2nd Ed., 1934.

phragm. The cannula in the duct was connected with a saline-filled rubber tube terminating in a glass dropper, the tip of which was adjusted to lie on a plane about 2 cm below the lowest level of the thoracic duct in the chest. The effluent was received into a graduated tube and its volume measured over 15-minute periods. Care was taken to keep the cannula free from clots. In 2 experiments chlorazol fast pink was administered (100 mg/kilo), rendering the lymph noncoagulable, and obviating the danger of intravascular clotting.

Flow measurements were made until the pulmonary edema that develops spontaneously in the heart-lung preparation had assumed massive proportions. No increase in flow was noted as the edema developed, but rather a tendency to decrease as fluid was lost from the blood stream into the lungs. Addition of saline or fresh blood to the reservoir restored the flow temporarily.

Any lymph collected from the thoracic duct in the preparation employed in these experiments can come only from the left side of the heart and the left lung. Moreover, since the bronchial vessels are

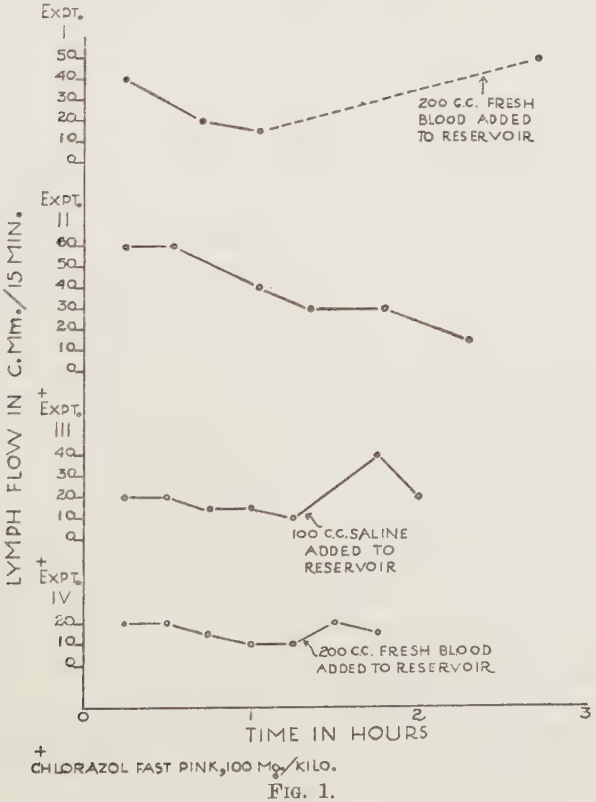

FIG. 1.

not being perfused, any pulmonary contribution must be made by the respiratory areas of the lung. But in view of the fact that flow was not augmented in spite of the development of massive pulmonary edema, it seems unlikely that alveolar tissue is provided with a mechanism for lymph drainage capable of conducting an appreciable flow.

11134

In vitro Conversion of Prontosil-Soluble to Sulfanilamide by Various Types of Microörganisms.*

Wesley W. Spink, Frank W. Hurd and Jean Jermsta.
(Introduced by C. J. Watson.)

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Fuller[1] has shown that both prontosil and prontosil-soluble are partly converted to sulfanilamide in the body. He also demonstrated *in vitro* that stannous chloride and sodium hydrosulphite could reduce prontosil-soluble to form sulfanilamide and amino-acetylamino-naphthol-disulphonic acid. When he gave prontosil-soluble to normal mice, only one-fourth appeared in the urine as sulfanilamide, whereas in mice infected with hemolytic streptococci and given prontosil-soluble, nearly one-half was excreted as sulfanilamide. Bliss and Long[2] succeeded in completely reducing prontosil-soluble *in vitro* with cysteine hydrochloride, and the resultant product proved to be actively bacteriostatic for hemolytic streptococci. They also observed that hemolytic streptococci and a strain of anhemolytic streptococcus were capable of decolorizing prontosil-soluble.[3]

Our purpose is to show that various types of bacteria are capable of converting prontosil-soluble to sulfanilamide. Under standard conditions, the facility with which this change takes place varies with different microörganisms. While such compounds as stannous chloride actually reduce prontosil-soluble to form free sulfanilamide, the mechanism is not as clearly defined whereby the conversion is

* Aided by a grant from the Graduate School of the University of Minnesota.

1 Fuller, A. T., *Lancet*, 1937, **1**, 194.

2 Bliss, E. A., and Long, P. H., *Johns Hopkins Hosp. Bull.*, 1937, **60**, 149.

3 Long, P. H., and Bliss, E. A., *Clinical Use of Sulfanilamide and Sulfapyridine and Allied Compounds*, 1939, 89.

accomplished by a biological system. It would appear that microorganisms achieve the same end result by reduction.

In seeking a standard method for quantitating this conversion power of bacteria, different procedures were tried. At first, a method described by Quastel and Whetham was used.[4, 5] "Resting cultures" of organisms were prepared by seeding a culture in broth, and allowing growth to proceed for 2 days. The broth culture was then centrifuged, the sediment washed 5 times in sterile distilled water, and aerated overnight to remove any oxidizable substance. Nitrogen was then bubbled through the concentrated culture to remove the oxygen. The sample was then diluted to a volume of 10 cc with distilled water. A one percent aqueous solution of glucose was used as the hydrogen donator. That concentration of an aqueous prontosil-soluble solution was used which yielded a 1 to 10,000 concentration of sulfanilamide when completely decolorized. Into a sterile pyrex test tube was placed 1 cc of culture, 1 cc of prontosil-soluble solution, 1 cc of glucose solution, 1 cc of 7.2 phosphate buffer, and 3 cc of distilled water. A control tube contained all the foregoing materials except that a molecular equivalent of an aqueous methylene blue solution was substituted for prontosil-soluble. The tubes were evacuated with a motor-driven vacuum pump, and then sealed in a gas-oxygen flame. The tubes were incubated in a water bath at 37°C. Under these conditions, several strains of staphylococci decolorized the prontosil-soluble and methylene blue solutions. The methylene blue solution was reduced in all instances within a very few minutes, whereas decolorization of the prontosil-soluble took several hours, and in some observations, days.

A more simple and effective method was finally adopted. Organisms were grown in peptone-broth media, with the exception of hemolytic streptococci, and *Streptococcus viridans,* in which cases brain-broth was used. The broth cultures were centrifuged, and the organisms resuspended in 5 cc of peptone-broth or brain-broth. The following were added to a sterile test tube: 0.1 cc of resuspended culture, 0.1 cc of aqueous prontosil-soluble solution (diluted to give a 1 to 10,000 concentration of sulfanilamide when completely decolorized) and 3.4 cc of broth. Control tubes were set up containing the same materials, except that methylene blue solution was substituted for prontosil-soluble in one tube, and in another tube organisms were omitted. The tubes were stoppered with cotton plugs, and incubated in a water bath at 37°C. The tubes

4 Quastel, J. H., and Whetham, M. D., *Biochem. J.*, 1925, **19**, 520.

5 Quastel, J. H., and Whetham, M. D., *Biochem. J.*, 1925, **19**, 645.

were examined at frequent time intervals for evidence of decolorization of the prontosil-soluble and methylene blue.

It was assumed that when complete decolorization of the prontosil-soluble took place, free sulfanilamide was one of the products formed. In a series of 7 observations, the contents of tubes showing complete decolorization were centrifuged, and the amounts of free sulfanilamide in the supernatant fluid were determined by the colorimetric method of Marshall and Litchfield.[6] This method depends upon diazotizing the sulfanilamide, and then coupling it with dimethyl-alpha-naphthylamine to form a purplish red dye. The spectral distribution courve of this dye solution was obtained with the use of the spectrophotometer, and was compared with the absorption curve for pure sulfanilamide, after it was diazotized and coupled in the same manner. The spectral distribution curves were the same for both solutions.

Twenty-two cultures of microörganisms were used in this study. The 6 strains of hemolytic streptococci belonged to Lancefield's Group A, and were supplied to us by Dr. F. Heilman of the Mayo Clinic. Several of the other cultures that were used were obtained from Dr. W. P. Larson of the Department of Bacteriology at the University of Minnesota.

The results are tabulated in Table I. It is noted that the organisms varied in their ability to decolorize prontosil-soluble. While

TABLE I.
Decolorization of Prontosil-soluble Solution by Various Microörganisms.

Organism	Total Hours of Incubation and Degree of Decolorization
Staph. aureus No. 24	93—complete
" " " 104	53—50%
" " " 114	18½—complete
" " " 25	90— "
" " " 107	21— "
" " " 16	22— "
Hem. strept. " 39	72—less than 10%
" " " 43	72— " " 10%
" " " 57	72— " " 10%
" " " 33	72— " " 10%
" " " 38	118—40%
" " " 56	20—complete
Strept. viridans	20— "
B. subtilis No. 1	24— "
" " " 2	92½—none
B. proteus " 1	18—complete
" " " 2	18— "
B. pyocyaneus No. 1	20— "
" " " 2	22— "
B. coli communis	27— "
" " *communior*	96—60%
D_4 (Unidentified Gram neg. Bac.)	16—complete

[6] Marshall, E. K., Jr., and Litchfield, J. T., Jr., *Science*, 1938, **88**, 85.

many strains produced the same change, the elapsed time necessary for complete decolorization varied considerably. When organisms, with the exception of the streptococcus, were suspended in a 2% aqueous solution of peptone instead of peptone-broth, there was a diminution in the speed and degree of decolorization.† A concentrated suspension of organisms yielded more complete decolorization than an unconcentrated suspension. Conversion was more rapid and complete in the presence of oxygen than under anaerobic conditions. Presumably, oxygen favored the growth of the organisms, which in turn produced a greater supply or greater activity of enzyme. Therefore, the optimum conditions for the *in vitro* conversion of prontosil-soluble depended upon a large inoculum of bacteria, the most favorable media for growth, and an available source of oxygen.

It is of interest that anaerobic conditions favored a more effective reduction of methylene blue. This difference in behavior between the 2 dyes may be partly explained on the basis that the decolorization of methylene blue is a reversible phenomenon, whereas it is irreversible for prontosil-soluble.

Completely decolorized prontosil-soluble solution yielded 10 mg per 100 cc of free sulfanilamide when determined by the method of Marshall and Litchfield.[6]

Summary. Different strains of bacteria vary in their ability to decolorize prontosil-soluble. It was shown that it was more difficult for microörganisms to decolorize prontosil-soluble than methylene blue.

11135

Functional Spinal Cord Regeneration in Adult Rainbow-Fish.

JOHN H. KEIL. (Introduced by Davenport Hooker.)

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Studies on the regenerative capacity of teleosts have given conflicting results. Koppanyi and Weiss[1] reported functional recovery and morphological reconstruction in the severed spinal cord of the adult goldfish. The return of function after spinal section in adults

† The difference in conversion-power of hemolytic streptococci, when compared to the other strains of microörganisms studied, may be due in part to the media used for the streptococcus, although brain-broth did not appear to affect the action of a strain of *Streptococcus viridans.*

1 Koppanyi, T., and Weiss, Paul, *Anz. d. Akad. d. Wissen, Wien.*, 1922, **7**, 206.

of this fish was further substantiated by Pearcy and Koppanyi.[2] However, Hooker[3] was unable to demonstrate either functional or morphological regeneration in adult goldfish after spinal section. Nicholas[4] failed to demonstrate morphological regeneration following section of the cord in Fundulus embryos, but Hooker[5] was able to show both functional and morphological restitution in the rainbow-fish when the cord had been severed during the first 4 days after "birth". Tuge and Hanzawa[6] proved both functional and morphological regeneration after spinal section in adult Japanese rice-minnows.

To further test regenerative capacity of the spinal cord in teleosts, the cord was transected in 132 adult rainbow-fish *(Lebistes reticulatus)*. Females were used because the males are too heavily pigmented for satisfactory translumination. The transluminated cord was cut with a narrow, thin-bladed knife under a binocular Greenough microscope, either just cephalic to the dorsal fin or at its caudal border.

Those which survived operation more than 24 hours (104 individuals) were observed and tested for reflexes at 24- to 48-hour intervals, for as long as 41 days in some cases.

The completeness of transection was evidenced by paralysis behind the lesion, drooping and loss of active motion in the caudal fin and appearance of the avoiding reaction described by Hooker.[5] To select fish with complete operations but to rule out extensive damage to structures other than the spinal cord, complete paralysis behind the lesion and a bilateral avoiding reflex were required. These conditions were fulfilled by 59 individuals, the others were considered to have incomplete operations (paralysis incomplete) or extensive damage to other structures (unilateral or absent avoiding reflex). The dorsal fin reflex, described by Tuge and Hanzawa[7] and considered by them to be pathognomic of spinal section in the Japanese rice-minnow, was discarded as a criterion because it can be evoked in normal rainbow-fish.

Of the 59 fish considered definitely spinal, 20 showed melanophoric expansion in the paralyzed area, 29 showed no color change, and in 10 there was a distinct loss in color.

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2 Pearcy, J. F., and Koppanyi, T., PROC. SOC. EXP. BIOL. AND MED., 1924, **22**, 17.

3 Hooker, Davenport, PROC. SOC. EXP. BIOL. AND MED., 1930, **28**, 89.

4 Nicholas, J. S., *Proc. Nat. Acad. Sci.*, 1927, **13**, 695.

5 Hooker, Davenport, *J. Comp. Neur.*, 1932, **56**, 277.

6 Tuge, H., and Hanzawa, S., *J. Comp. Neur.*, 1937, **67**, 343.

7 Tuge, H., and Hanzawa, S., *Sci. Repts. Tohoku, Imp. Univ., Biol.*, 1935, **10**, 589.

Return of function was evidenced by the appearance of an occasional flexion of the tail and either normal spreading of the caudal fin or its active motion (side to side fanning or its minute undulating motion constantly present in the normal fish). At first these signs were present only for short periods, following stimulation or when appearing spontaneously. These activities gradually became more constant, more forceful and better coördinated. In a few cases, the behavior ultimately closely approached that of the normal fish. The interval between operation and the first evidence of returning function varies greatly. In 3 fish such evidence appeared on the third day after operation; in 31 from the fourth to the sixth day; in 12 between the seventh and eleventh day. In addition, 13 others, observed for 7 days or less and which were among those sacrificed at intervals after operation for morphological study, showed no evidence of returning function before terminal anesthesia and fixation.

The evidence afforded by these observations indicates that the adult female of the rainbow-fish, *Lebistes reticulatus,* exhibits a high capacity for functional restoration following section of the spinal cord.

11136 P

Changes of Choline Esterase at End Plates of Voluntary Muscle Following Section of Sciatic Nerve.

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Choline esterase, present throughout the body, exists in especially high concentrations at the end plates of striated muscle, in the synapses both of the central nervous system and of the sympathetic ganglia.[1, 2] The concentration in these 3 foci is sufficiently high to satisfy the requirements of the theory that acetylcholine (ACh) may be involved in the transmission of nerve impulses from neurone to neurone or from neurone to striated muscle fibre. The alterations in enzymatic concentration which occur after the nerve endings disappear have been investigated.

* Now at the Laboratory of Physiology, Medical School, Yale University.

1 Marnay, A., and Nachmansohn, D., *J. Physiol.*, 1938, **92**, 37.

2 Nachmansohn, D., *Bull. Soc. Chim. Biol.*, 1939, **21**, 761.

It has been found in guinea pigs that section of the sciatic nerve is followed by a rise in *concentration* of choline esterase in the gastrocnemius muscle.[3] The Q.Ch.E. (mg ACh hydrolyzed per hour by 100 mg of fresh tissue) depends to a large extent on the number of end plates per unit of weight because of the high concentration of enzyme at these points. It was thought that the increase in choline esterase might be due to the decrease in volume of muscle fibers, leading to an increase in the number of fibers per unit of weight and consequently in number of end plates or more precisely "sole-plates" which persist after the nerve endings have disappeared. If the volume of fibers decreased and the Q.Ch.E. increased at the same time to the same extent it would imply that the enzyme concentration at the end plates remained practically the same.

Determinations of the volume of fibers, carried out 1-3 weeks after denervation show that the decrease fully accounts for the apparent increase of enzyme concentration. The rise of concentration actually is not quite as great as the corresponding diminution of volume.

The *total amount* of enzyme in the muscle decreases, the loss of weight being more important than the increase in concentration. Subtraction of the amount of enzyme present in muscle fibers from the total amount permits an approximate estimation of the amount of enzyme present at the end plates after section of the sciatic. During the first 2 weeks at the end of which nerve endings have disappeared, no measurable decrease of the enzyme concentration at the end plates occurs. Since during this time the enzyme concentration in the peripheral part of the cut sciatic falls by 50%, it can be assumed that with the disappearance of nerve endings some enzyme disappears, but that the amount is small compared with the large total amount of enzyme present at the end plates *outside* the nerve endings. After 3-4 weeks the enzyme at the "sole-plates" has decreased by about 30-40%. But a high concentration still persists and remains there for months.

The persistence of the enzyme at the end plates of denervated muscle can be demonstrated by a direct method based on a special arrangement of the nerve in the interior section of the gastrocnemius: The nerve spreads through nearly the whole muscle at one level only, situated in the midst of the muscle except for its entrance and its termination. In the middle third of this muscle all nerve fibers as well as the endings are situated in the middle zone. If this third is cut in slices with a freezing microtome the slices near the

[3] Marnay, A., and Nachmansohn, D., *C. R. Soc. Biol. Paris*, 1937, **126**, 785.

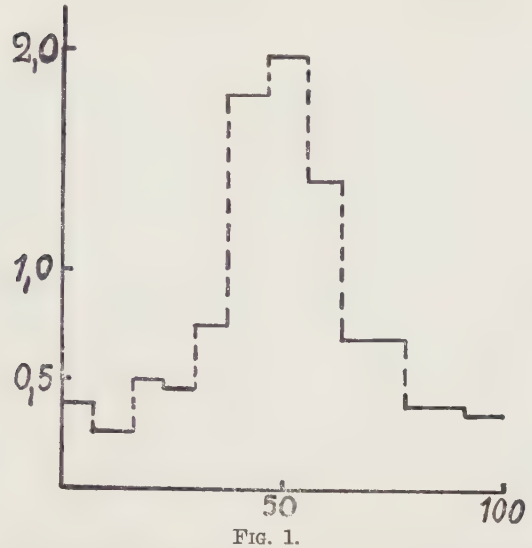

FIG. 1.

Concentration of choline esterase in the middle portion of the interior section of a guinea pig's gastrocnemius cut in 11 slices of similar thickness and weight. Each horizontal line corresponds to one slice and indicates its weight in % of the total weight. Abscissæ: Region from which the tissue was obtained in terms of order of consecutive slices. Point 50 corresponds to the center region where the nerve endings are situated. Ordinates: Q.Ch.E.

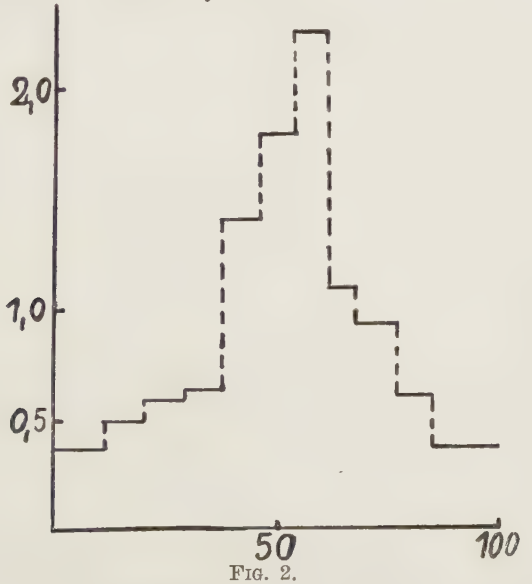

FIG. 2.

Concentration of choline esterase in the middle portion of the interior section of a guinea pig's gastrocnemius cut in 11 slices of similar thickness and weight, 14 days after section of the sciatic. Each horizontal line corresponds to one slice and indicates its weight in % of the total weight. Abscissæ: Region from which the tissue was obtained in terms of order of consecutive slices. Point 50 corresponds to the center region where the nerve endings are situated. Ordinates: Q.Ch.E.

upper and lower surfaces are practically free of nerve endings whereas those of the middle zone contain a large number. Determinations of choline esterase in these slices show a high concentration in the middle zone as compared with low concentration in the 2 nerveless zones. Fig. 1 gives the results obtained from the slices which are arranged in the order in which they were cut. The results confirm previous observations on frog sartorius. After denervation and the disappearance of nerve endings the concentration of the enzyme in the middle zone remains as high as before (Fig. 2).

The results indicate that the main bulk of the enzyme at the end plates is located outside the nerve endings. With their disappearance only a small amount disappears, which cannot be estimated. Later the enzyme concentration decreases to a certain extent but still remains high. Regeneration of the muscle fibers leads to an increase both of the concentration and of the total amount of the enzyme.

Bruecke observed a decrease of choline esterase in the superior cervical ganglion of cats after section of the preganglionic fibers and concluded that there was a high concentration inside the nerve endings.[4] These observations were qualitative estimations. Quantitative determinations confirming Bruecke's results, show that during the time when the nerve endings disappear the choline esterase decreases by about 60% (Fig. 3), which apparently indicates a high

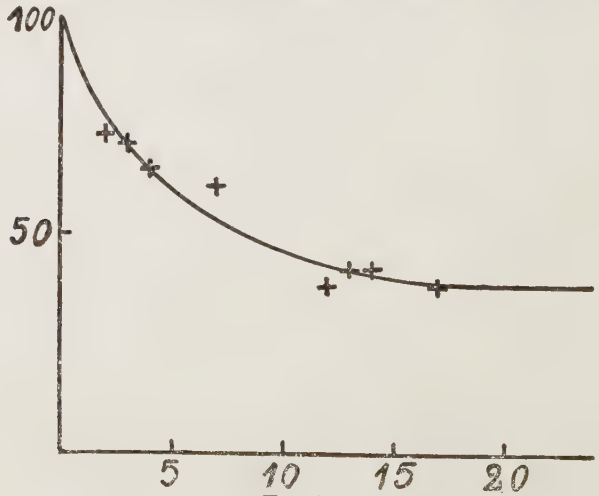

FIG. 3.

Decrease of choline esterase in the superior cervical ganglion of cats, after section of the preganglionic fibres. Abscissæ: Days after section. Ordinates: Amount of enzyme in % of the initial amount.

[4] Bruecke, F. v., *J. Physiol.*, 1937, **89**, 429.

amount localized inside the nerve endings. But then the enzyme power remains constant. After 5 weeks the same value was obtained. The Q.Ch.E. falls from the normal 40-60 to 20-25. This latter value is still very high. It is difficult to explain such a high value by the presence of the remaining fibers and cell bodies. There is reason to assume that considerable fraction of this enzyme is concentrated around the endings of preganglionic fibers and persists there as in the case of striated muscle. Even a fraction of the persisting enzyme if localized at the synapses would be sufficient to split during the refractory period the amount of ACh liberated at this ganglion by stimulation of preganglionic fibers.

The main difference between neuro-muscular junctions and ganglionic synapses seems to be that in the latter a greater fraction of the enzyme is localized inside the nerve endings. The disparity may be related to the powerful end arborization of preganglionic fibers which is not paralleled by that of the motor nerve endings of guinea pigs. The increase of the Q.Ch.E. of preganglionic fibers from 5.0 to a several times higher value at the nerve endings in the ganglion may indicate that the enzyme is localized near surfaces.

11137 P

Effect of Urine from Gastrectomized and Duodenectomized Dogs on Gastric Secretion.

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(Introduced by T. L. Patterson.)

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Previously we reported that from the urine of normal individuals there can be extracted a substance which inhibits gastric secretion when administered intravenously[1, 2] but not when administered subcutaneously[2] in the same dose. Ivy and his coworkers[3] and Necheles[4]

* With the assistance of R. O. Recknagel and H. M. Podolsky.

1 Friedman, M. H. F., Recknagel, R. O., Sandweiss, D. J., and Patterson, T. L., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 509.

2 Sandweiss, D. J., Saltzstein, H. C., and Farbman, A. A., Detroit Physiological Soc., March 3, 1938; A.M.A., San Francisco Meeting, June 17, 1938; also *Am. J. Digest. Dis.*, 1939, **6**, 6.

3 Gray, J. S., Wieczorowski, E., and Ivy, A. C., *Science*, 1939, **89**, 489.

4 Necheles, H., personal communication to Dr. D. J. Sandweiss, June 30, 1938; also Necheles, H., Hanke, M. E., and Fantl, E., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 618.

have also studied the inhibition of gastric secretion by extracts of normal urine. An investigation was undertaken to determine the source or the mechanism of the body which is responsible for the elaboration of the active secretory depressant principle found in the urine. The possibility that the secretory depressant is formed in the gastrointestinal tract and is concerned with the autoregulation of gastric secretion is obvious. That the substance may be enterogastrone excreted in the urine was the suggestion put forward by Ivy and coworkers.[3]

We reasoned that if the gastric secretory depressant is of gastric origin, this would be shown in a study of urine extracts prepared from patients with extensive inoperable carcinoma of the stomach or from patients with pernicious anemia. However, both types of urine extract inhibited gastric secretion just as did the normal urine extract.[5] Accordingly, we resorted to the preparation of urine extracts from duodenectomized and from gastrectomized dogs. The collection of urine from these dogs was begun 3-4 weeks after the operation. This long interval between the operation and collection of urine insured depletion of possible stores of the substance in the body as well as obviated the early depressing effects of the operation.

Method: The benzoic acid adsorption procedure of Katzman and Doisy[6] for obtaining the gonadotropic hormone in pregnancy urine (Antuitrin-S) was employed in preparing the human and canine urine extracts used in our study. Assays were made on vagotomized dogs under nembutal anesthesia. Gastric juice was obtained by fistula from the whole stomach, contamination by oesophageal and intestinal secretions being prevented by ligation of the oesophagus and pylorus. Gastric secretion was stimulated by hourly subcutaneous injections of histamine phosphate (0.1 mg per kilo per hour). Urine extracts were administered intravenously at the end of the second hour. Because dog's urine is more concentrated than human urine, the extracts were administered in terms of original volume of urine rather than weight of extract. Rectal temperatures were taken at intervals throughout the course of the experiment, since "urine preparations under certain conditions cause a rise in body temperature" as "they contain a pyrogen."[7]

Results. Extracts from both normal human and dog urine, given 2 hours after the initial hourly injection of histamine, inhibited

[5] Friedman, M. H. F., Sandweiss, D. J., Recknagel, R. O., and Patterson, T. L., *Anat. Record,* 1939, **75**, Sup., 53.

[6] Katzman, P. A., and Doisy, E. A., *J. Biol. Chem.,* 1932, **98**, 745.

[7] Ivy, A. C., personal communication, Oct. 31, 1939.

markedly the secretion of gastric juice. Inhibition commenced within 45 minutes and lasted for 3 or 4 hours. The percent inhibition during the 3-hour period following the administration of extract is shown in Table I. It is seen that the extracts of urine from duodenectomized and from gastrectomized dogs also inhibited secretion. The extracts were without effect on blood pressure and their inhibitory effects on gastric secretion were quite independent of the course of body (rectal) temperature.

TABLE I.

The inhibition of gastric secretion during the 3 hours following administration of urine extract is expressed as percent of the secretion in control experiments during the same period.

Nature of urine	No. of experiments	% inhibition
Control (no urine given)	9	0
Human, Normal	7	67.5
Dog, Normal	5	63.0
" Gastrectomized	7	45.9
" Duodenectomized	4	56.0
Human, Heat-inactivated	3	1.5

Conclusion. An extract can be prepared from the urine of normal dogs which inhibits gastric secretion. The inhibitory principle is still present in the urine after removal of either the stomach or duodenum.

11138

Hormonal Inhibition of Lactation.*

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(Introduced by C. W. Turner.)

From the Departments of Dairy Husbandry, New Jersey Agricultural Experiment Station and Nebraska Agricultural Experiment Station.

In a previous communication[1] it was reported that the daily injection of 100 r.u. of a gonadotropic principle during the first 5 days of the lactation period in the rat caused no inhibition of lactation. Larger dosages and dosages over a longer period of time re-

* Contribution from the Departments of Dairy Husbandry, Journal Series paper of the New Jersey Agricultural Experiment Station, and Nebraska Agricultural Experiment Station, Journal Series No. 256.

1 Hathaway, I. L., Davis, H. P., Reece, R. P., and Bartlett, J. W., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 214.

duced the rate of growth of the young 22 to 33%. Earlier work[2, 3, 4] showed that estrogens were less effective in inhibiting lactation in the ovariectomized rat than in one with ovaries intact. These results stimulated interest in attempting to determine whether or not the effectiveness of estrogens in inhibiting lactation could be augmented through simultaneous administration of a gonadotropic principle and, if possible, the route of the inhibitory action.

Fifty-six lactating rats and their litters were used in this study. The number of young in the litters was fixed at 6, and daily weighings were made. The diet fed was the same as that reported in a previous paper.[1] It was decided to use as a criterion for the inhibition of lactation the failure of a litter to gain weight on 3 successive days. Seventeen lactating rats, designated hereafter as Group I, were injected daily with 200 r.u. of a gonadotropic principle from pregnant women's urine (Antuitrin-S†) plus 100 r.u. of an estrogen (Progynon-B†). Group II contained 17 lactating rats that received daily 100 r.u. of Progynon-B. In Group III 5 lactating rats were injected daily with 200 r.u. of Antuitrin-S. Seventeen lactating rats, Group IV, served as controls. When the young of a lactating rat in Group I failed to gain weight on 3 successive days they were removed, the lactating rat was injected with one gamma of colchicine per gram of body weight and sacrificed 9½ hours later. For comparative purposes a lactating rat from each of Groups II and IV, in the same stage of the lactation period as the rat from Group I, was given similar treatment. No experimental lactating rats received more than 10 injections.

The ovaries and pituitaries were removed and weighed and the latter assayed for their lactogen content by injecting the tissue intradermally over the crop glands of common pigeons. Mammary glands were removed and fixed for study.

In Group I, 16 of the 17 litters failed to gain weight on 3 successive days, 4 pups having died before reaching this stage. Five of the 17 litters in Group II failed to gain weight on 3 successive days, one pup having died before reaching this stage. In Groups III and IV none of the litters failed to gain weight. On the 11th day of the lactation period the average weight of the remaining young in the

[2] Anselmino, K. J., and Hoffman, F., *Zentralbl. f. Gynak.*, 1936, **60**, 501.

[3] Reece, R. P., and Turner, C. W., *Mo. Agr. Exp. Sta. Res. Bul.*, 1937, 266.

[4] Folley, S. J., and Kon, S. K., *Proc. Roy. Soc., B*, 1938, **124**, 476.

† We are indebted to Dr. Oliver Kamm of Parke, Davis and Company for the Antuitrin-S and to Dr. Erwin Schwenk of the Schering Corporation for the Progynon-B used in this study.

several groups was as follows: Group I, 9.7 g; Group II, 12.8 g; Group III, 17.3 g; and Group IV, 18.8 g.

The ovaries of the rats in Group I (268.4 mg) were significantly heavier than those of the rats in Group II (73.6 mg) and Group IV (69.8 mg) while the pituitary glands from the rats in Group I (14.0 mg) and II (14.2 mg) did not differ significantly yet they were significantly heavier than the pituitary glands of rats in Group IV (9.4 mg).

The pituitary glands of the rats in Group I contained more lactogen than did those of Groups III and IV and nearly the same as did those in Group II. Pituitary glands from rats in Group II contained more lactogen than did the glands from rats in Groups III and IV while the lactogen content of the glands of rats in Groups III and IV was similar. The results are summarized in Table I.

Microscopic examination of the mammary glands of animals in Groups IV (controls) and III (Antuitrin-S) revealed similar conditions. Mitotic figures were rarely observed and the lumina of the alveoli were distended with milk. Epithelial cells exhibiting arrested mitosis were occasionally found in the mammary gland of rats in

TABLE I.
Hormonal Inhibition of Lactation.

Group*	No. of comparisons	Avg body wt following parturition, g	Avg wt of remaining young on following day of lactation cycle, g			Avg wt of pituitary, mg	Avg. No B.U.† per pituitary	Ovarian wt, mg
			1	6	11			
I	17	236	5.6	8.2	9.7	14.0	27	268.4
IV		242	5.6	11.0	18.8	9.4	23	69.8
I	17	236	5.6	8.2	9.7	14.0	26	268.4
II		231	5.7	10.4	12.8	14.2	27	73.6
II	17	231	5.7	10.4	12.8	14.2	28	73.6
IV		242	5.6	11.0	18.8	9.4	20	69.8
III	5	245	5.7	10.1	17.3	11.7	17	220.1
IV		243	5.5	10.9	19.8	10.6	18	70.6
III	5	245	5.7	10.1	17.3	11.7	15	220.1
I		242	5.6	8.2	8.5	16.0	23	288.1
III	5	245	5.7	10.1	17.3	11.7	19	220.1
II		244	5.8	11.0	12.2	16.1	28	72.6

*Group I received daily 200 r.u. of Antuitrin-S plus 100 r.u. of Progynon-B; Group II injected daily with 100 r.u. of Progynon-B; Group III injected daily with 200 r.u. of Antuitrin-S; and Group IV were the controls.

†B.U.—Bird units.

) Pituitary glands assayed in the same group of birds.
)

Group II (Progynon-B). The lumina of the alveoli were filled with milk. In marked contrast many mitotic figures were observed in the gland parenchyma of rats in Group I. The lumina of the alveoli usually contained very little milk, and in a few cases they were entirely absent.

Conclusions. The effectiveness of estrogens in inhibiting lactation in the rat can be increased through simultaneous administration of a gonadotropic principle from pregnant women's urine. Many cells in mitoses are observed in the mammary gland parenchyma of rats so treated. It is believed that this is the mechanism of the inhibitory action of these hormones on lactation. That is, one would not expect a proliferating gland to secrete as efficiently as a non-proliferating gland. The administration of an estrogen plus a gonadotropic principle to lactating rats augments the lactogen content of the pituitary gland.

11139 P

Influence of Heredity and Environment upon Number of Tumor Nodules Occurring in Lungs of Mice.

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In previous publications it has been shown that the occurrence of lung tumors in mice is influenced by environment and also by heredity[1] and that probably, in the strains studied, a single dominant Mendelian factor is concerned.[2, 3] We emphasize, however, that our conception as to number of genes may be affected by further study of environmental conditions. The present paper deals with the influence of heredity and environment upon degree of susceptibility among mice with tumor.

The measure used, in this instance, to determine degree of susceptibility, is the number of tumor nodules found in each individual. Although the counts covered only those nodules visible on the surface of the lung and obviously may not represent the total number possessed by the mouse, they are sufficiently accurate for our purpose. In Table I, comparisons are made of the number of lung

[1] Lynch, C. J., *J. Exp. Med.*, 1927, **46**, 917; 1931, **54**, 747; *Occas. Publ. Am. Assn. Ad. Sci.*, 1937, **4**, 22.

[2] Bittner, J. J., *Pub. Health Rep.*, 1938, **50**, 2197.

[3] Lynch, C. J., *Third Internat. Canc. Cong.*, in press.

TABLE I.

Mice Belonging to Various Groups Arranged According to the Number of Tumor Nodules Occurring in the Lungs of Each Individual.

Tumor	Group of mice	% tumor	No. of Nodules																					
			0	1	2	3	4	5	6	7	8	9	10	1-10	11-20	21-30	31-40	41-50	51-60	61-70	71-80	81-90	91-100	11-100
Spontaneous	Strain 1194	4.4	1676	67	8	2				1				78										0
	" Swiss-8	43.9	384	176	69	27	19	4	1	2	1			299	1									1
Induced	" 1194	20.5	290	66	3	4	1			1				75										0
	" Swiss-8	100.0				2				2		1	1	6	17	16	6	3	2	1				45
	Backcross to 1194	68.3	13	9	3	3	3	1	1	1		1	2	24	4									4
	F_2	91.7	3	1	4	6	2			1	1	2		17	6	4	2	3			1			16
	Backcross to Swiss	100.0				1	1						1	3	8	8	9	4	5	3		2	1	40

nodules occurring in mice from 2 strains living under ordinary laboratory conditions and also in mice from the same strains after treatment with tar. Various generations from a cross between these strains are also compared after tarring. Strain 1194 is a low tumor strain which has been under observation in our laboratory for many years, and this sample, which is characteristic, has only 4.4% lung tumor among mice of all ages. Of 78 individuals with tumor, the very large majority had a single nodule though one mouse had as many as 7. On the other hand, Strain Swiss-8 has a higher percentage of tumor. In the group of 684 mice represented in the table, the tumor incidence was 43.9%. As to number of nodules, the range per individual was somewhat greater than in the first strain. Also the number of individuals with multiple nodules was, in a statistical sense, significantly greater among the Swiss-8 than among the 1194 mice ($\chi^2 = 19.9$ and P is $<.01$). The difference between strains is clear.

It has been shown previously that certain treatment with chemicals increases the percentage of tumor mice though not equally so in all strains[1] and it appears from the present experiment that the number of nodules per individual also may be increased by this means. When Strain 1194 was tarred, the tumor incidence increased from 4.4 to 20.5% even though the treated mice were killed rather early (12-13 months of age). In this strain the number of nodules was scarcely affected; the range in nodule number was about the same as in the non-tarred mice of this strain and about the same percentage of the tumorous animals had a single focus of malignant growth. The effect of tarring upon the Swiss-8 strain was more marked; of 51 animals treated, all had tumor, all had multiple growths and the large majority had more than 10. Evidently the change in external factors caused a striking shift in the frequency of the nodules, possibly by lowering the threshold of susceptibility. While the Swiss-8 strain may not have been genetically uniform at the time this experiment was performed, there can be no doubt that the tar had an effect. The difference between the Swiss-8 and the 1194 strain is more pronounced when the mice are tarred and under this condition the influence of both heredity and environment can be seen.

In an attempt to get further evidence of the genetic effect besides that shown by the contrast between strains, these strains were crossed. None of the first hybrid generation from this cross was treated; however, in similar experiments to be reported later, the F_1 were intermediate as to number of nodules shown. Mice from the hybrid generation were crossed back to the low strain as well as to the high strain and were also inbred to produce an F_2 generation. Representa-

tives from the 3 groups thus obtained were subjected to tarring. The tumor rates may have been somewhat affected by parental variability. When the cross was made to the low tumor strain the range in number of nodules per mouse approached that given by the low tumor parents and the class with a single tumor was the mode. On the other hand, crossing back to the high tumor strain resulted in progeny with a range in number of tumor foci as great—even slightly greater than that of the parental Swiss-8 strain. The F_2 generation, which was intermediate between the backcross groups in respect to percentage of tumor mice, was intermediate also in regard to range and distribution in number of nodules. If a mathematical comparison is made on the basis of the possession of more than 10 nodules, it is found that a significantly greater number of mice with more than 10 occurs among the F_2 than among the mice obtained by backcrossing to the low tumor strain No. 1194 ($\chi^2 = 7.22$ and P is $<.01$), and that a significantly smaller number of such individuals occurs in the F_2 than among the mice from the backcross to the Swiss-8 ($\chi^2 = 18.12$ and P is $<.01$). It is apparent that multiplicity of tumor nodules is influenced by heredity. Previous evidence indicated that probably a single dominant gene is concerned in the inheritance of susceptibility to lung tumor. While heterozygosity of this gene as well as uncontrollable extrinsic variables may affect, to some extent, the degree of susceptibility, it is possible that additional genes, as well as certain known environmental conditions, may influence the number of tumor nodules shown by each mouse.

Summary. Evidence is presented showing that degree of susceptibility to lung tumor in mice, as measured by the number of nodules occurring in the individual, is affected by both heredity and environment. The number of genes concerned has not been determined.

11140

Prothrombin Levels and Synthetic Vitamin K in Obstructive Jaundice of Rats.*

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Through dietary studies, the chick has helped to solve a number of problems concerning the chemical nature of vitamin K and its relation to the manufacture of prothrombin. However, there still exist many important questions concerning the minimal vitamin requirements, the absorption of the vitamin, the mechanisms by which it is utilized, and the rôle of the liver in the production of prothrombin. Clinical problems in man involve not merely the normal dietary requirements, but also the question of absorption from the intestine, and the problem of having adequate amounts of bile in the intestine to aid in this absorption.

To analyze a number of the problems it is desirable that an experimental deficiency be created in animals by excluding bile from the intestine, and not by dietary procedures alone. It is also desirable that certain of these studies be made on mammals, rather than on birds in order to approach more closely the physiological conditions obtaining in man.

Greaves[1] has shown that a bleeding tendency can be produced in the rat by a combination of dietary regulation and bile duct ligation and that this disorder can be cured by administration of crude concentrates of vitamin K. We have found that the prothrombin level falls both rapidly and regularly to low levels, and the response to vitamin K is sufficiently uniform that such an animal represents a valuable test animal for study of the problems outlined above. We wish to present data concerning the prothrombin levels before and after treatment with vitamin K.

Experimental Procedure. In some cases, the rats were selected at the time of weaning; in other cases young adult rats were used. In order to deplete the reserves of vitamin K in the body, the animals

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The phthiocol and the 2-methyl-1,4-naphthoquinone used in these experiments were prepared through the courtesy of Dr. George H. Coleman and Dr. Donald W. Kaiser, Department of Chemistry, State University of Iowa.

1 Greaves, Joseph D., *Am. J. Physiol.*, 1939, **125**, 429.

were placed on a diet known to be low in this vitamin. The diet was prepared as follows: cane sugar 472 g, casein 480 g, brewer's yeast 120 g, salt mixture[2] 68 g, lard 330 g, cod liver oil 30 g.

After the rats had been on this diet for 7 to 8 weeks, the common bile duct was ligated, under ether anesthesia, and the incision was closed with silk sutures. The rats weighed between 250 and 350 g at the time of operation in most instances. A few animals operated before they reached a weight of 250 g gave similar results.

Blood samples (0.8 cc) were drawn from the jugular vein into a 1 cc syringe containing 0.23 cc potassium oxalate (1.85%). The oxalated blood was centrifugalized in a small tube, and the prothrombin content of the plasma was determined by the 2-stage method of Warner, Brinkhous and Smith.[3,4] By this microtechnic, prothrombin determinations can be made several times on the same animal without producing a significant degree of anemia. We prefer jugular puncture to cardiac puncture, because the latter is far more likely to be followed by fatal hemorrhage, especially in case the prothrombin level is low.

Both phthiocol (0.2%) and 2-methyl-1,4-naphthoquinone (200 gamma per 100 cc) were dissolved in isotonic phosphate buffer solutions (pH 7.2).[5] Phthiocol solutions were not injured by sterilization in the autoclave, but 2-methyl-1,4-naphthoquinone suffered marked loss of activity. In our earlier experiments we injected the vitamin preparations without sterilization. Recently, we have found that sterilization of solutions of 2-methyl-1,4-naphthoquinone can be effected with the use of "fritted" glass filters.

Results. With deficient diet alone, it is possible to deplete the vitamin K reserves, as Greaves[1] has pointed out, but rarely does the prothrombin level fall far below the normal. It would appear that in mammals, the vitamin K synthesized in the intestine by bacteria is sufficient to prevent a marked fall in the prothrombin level. This preliminary depletion of vitamin K reserves is essential if one is to bring about a bleeding tendency promptly by ligation of the bile duct. In the undepleted rat, bile duct ligation results in a very gradual decrease in the plasma prothrombin level, and even after 3 weeks, the prothrombin level is still 20% to 40% of normal. By this time, we

2 Hubbell, R. B., Mendel, L. B., and Wakeman, A. J., *J. Nutrition*, 1937, **14**, 273.

3 Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, **114**, 667.

4 Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Exp. Med.*, 1937, **66**, 801.

5 Smith, H. P., Ziffren, S. E., Owen, C. A., Hoffman, G. R., and Flynn, Joseph E., *J. Iowa State Med. Soc.*, 1939, **29**, 377.

TABLE I.
Decrease in Prothrombin After Bile Duct Ligation.

Hr post-operative	Prothrombin % of normal Rat No. 1	2	3	4
6	82	100	100	84
24	73	57	72	42
48	35	21	61	5
72	10		27	5

find that general debility and cirrhosis of the liver render the animal unsuitable for these studies.

Table I shows the rate at which the prothrombin level falls in the depleted rat following ligation of the bile duct. During the first 24 hours, the prothrombin level falls only moderately in the majority of animals. By the end of 72 hours the level is typically 10% to 25% of normal. At the 10% level the rats invariably bleed unduly from venipuncture wounds or from small scratches, and at the level of 20% to 25% this is frequently the case.

The response to treatment with vitamin K is shown in Table II. Two daily intraperitoneal doses of phthiocol, 1 mg each, brought the prothrombin level, first from 16% to 47%, and then to 91% of normal. We have administered daily doses of 0.5 mg, and in some cases a definite rise occurred; in others it did not. This would indicate that 0.5 mg represents roughly the daily maintenance requirement, and that the prothrombin level falls into the danger zone if the intake is much less than this.

A typical response to 2-methyl-1,4-naphthoquinone is shown in

TABLE II.
Prothrombin Response to Vitamin K.
(Intraperitoneal Injection.)

Days post-operative	Prothrombin % of normal	Vitamin K
Response to phthiocol (2-methyl-3-hydroxy-1,4-naphthoquinone)		
3	16	1 mg
4	47	1 "
5	91	—
Response to 2-methyl-1,4-naphthoquinone		
3	10	2 γ
4	61	2 γ
5	82	—
Response to a single large dose of 2-methyl-1,4-naphthoquinone		
3	5	20 γ
4	81	—
5	78	—
6	59	—
7	20	—

the second section of Table II. Other experiments, not recorded here, agree with this one in that 2 daily doses of 2 gamma each bring about almost complete recovery in the prothrombin level within 48 hours.

In a number of experiments the vitamin preparations were injected intravenously rather than intraperitoneally. In these experiments the response to both the phthiocol and the 2-methyl-1,4-naphthoquinone was essentially the same as that obtained by the intraperitoneal route of administration.

In comparing the results obtained with the two vitamin preparations, it is evident that under the conditions obtaining in our experiments, the 2-methyl-1,4-naphthoquinone has approximately 500 times the activity of phthiocol. This differs moderately from the ratio of 1000 to 1 which Ansbacher[6] found on using the deficient chick as an assay animal.

We have conducted a number of experiments in which the same rat was used for several successive test doses of vitamin K. This permits more accurate control than when different animals are employed. It is especially valuable in comparing different doses of a compound or different modes of administration.

Table II shows that when a single 20 gamma dose of 2-methyl-1,4-naphthoquinone was given by the intraperitoneal route, the prothrombin level rose in 24 hours to 81% of normal. It is of particular interest that this level was not long maintained, for within 3 days the prothrombin was reduced to 20% of normal. In another case we gave a much larger dose of vitamin on each of 2 successive days and found that the normal level was maintained for a number of days. It would appear that, although the avitaminous animal can efficiently utilize very small amounts of the vitamin to reëstablish the integrity of the disordered clotting mechanism, storage of the vitamin is far more difficult to accomplish. Thus the depleted animal can build up normal stores only when the vitamin is assimilated in large amounts, or perhaps with a smaller excess over daily needs, if continued over a long period of time. This observation helps to explain the clinical fact that in cases of biliary tract disease the rise in prothrombin on giving vitamin K is often quite transient, making it necessary to give the vitamin repeatedly during the period immediately preceding and immediately following operation. It is probable that the dosage ordinarily given to patients is not adequate to replenish the much-needed stores.

Summary. Rats maintained on a diet low in vitamin K, and having obstructive jaundice, were used to indicate the vitamin K

[6] Ansbacher, S., *Science*, 1939, **90**, 215.

potency of phthiocol and of 2-methyl-1,4-naphthoquinone. Data also are given to indicate the approximate maintenance requirement of this animal. Evidence at hand indicates that considerable amounts of vitamin K can be stored, provided the deficient animal is given large doses of vitamin K, or perhaps with smaller doses over a long period of time. The clinical implications of this are pointed out.

11141

Active and Passive Immunization Against the Virus of Malignant Panleucopenia of Cats.

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A common, acute infectious disease of cats characterized by intranuclear inclusion bodies, profound depression of the number of lymphocytes and granulocytes of the blood and an extensive aplasia of lymphoid tissue and of the bone marrow including erythropoietic elements has recently been described by Hammon and Enders[1, 2, 3] who showed that the etiologic agent was filterable.* The disease is probably identical with that previously reported by Lawrence and Syverton.[4] The experiments recorded here in detail† had as their objectives the determination of whether or not active immunization against subsequent inoculation of the virus could be effected in the natural host by means of formalinized suspensions of organs from infected cats and whether or not the immunity which had been found to result from an attack of panleucopenia could be passively transferred to susceptible animals by means of the blood serum.

Active Immunization. The data obtained in 2 experiments are pre-

1 Hammon, W. D., and Enders, J. F., *J. Exp. Med.*, 1939, **69**, 327.

2 Hammon, W. D., and Enders, J. F., *Ibid.*, 1939, **70**, 563.

3 Enders, J. F., Third International Congress for Microbiology, New York, 1939, Abstracts of Communications.

* Communications which we have received from a number of investigators indicate that the disease is present in Canada, Germany, Russia, and possibly South America.

4 Lawrence, J. S., and Syverton, J. T., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 914.

† Certain of the data included here were briefly described at the meeting of the Fourth International Congress for Microbiology held at New York, September 9-14, 1939.

TABLE I.
Immunization-Experiments with Formalinized Vaccine.

	Vaccinated						Controls				
Exp.	Kitten No.	Dose of vaccine	Mode of test-infection	Leuco-penia	Wt loss	Out-come	Kitten No.	Mode of test-infection	Leuco-penia	Wt loss	Out-come
1	206	2 doses, 2 cc (1)	Intraäbdominal	0	0	S	210	Intraäbdominal	8,300 (5) +	+	S
	207	,,	,,	0	0	S	211	,,	0 (3)	+	S
	208	,,	,,	0	0	S	212	,,	+	+	D
	209	,,	,,	0	0	S	213	,,	5,000 (5) +	+	S
2	236	2 doses, 2 cc (2)	Exposed and intraäbdominal	0	0	S	238	Exposed (4)	+	+	D
	237	,,	,, ,, ,,	0	0	S	239	Exposed and intraäbdominal	450 (5) +	+	S
						0 D					2 D
Total	6			0	0	6 S	6		5	6	4 S

(1) 0.3% formalin added to a 10% suspension of spleen from an infected animal, stored 4 days at room-temperature, then 7 days at 5°C until first used. Given subcutaneously.
(2) 0.3% formalin added to a 10% suspension of spleen and liver from an infected animal, stored 5 days at room-temperature, then 5½ months at 5°C. Given subcutaneously, 1 cc at 2 different sites.
(3) Developed progressive anemia with marked increase in erythrocytic fragility.
(4) This animal became infected through some unknown exposure and became ill, exposing in turn the vaccinated kittens and the other control before their inoculation with virus.
(5) Lowest leucocyte count obtained—counts made daily following inoculation.

sented in Table I. The kittens employed were secured from distant rural areas to avoid encountering a high percentage of naturally immune animals as is found normally in those collected in and near an endemic center such as Boston. The vaccine prepared according to the procedure included in the notations of the table was given in 2 doses at an interval of 7 days. The test dose of virus which consisted of 1 cc of a 10% suspension in infusion-broth, of spleen or lymph-node from animals dying or dead as a result of the specific infection, was administered 7 days following the last injection of vaccine. Normal kittens, which were litter-mates of the vaccinated animals, served as controls. These received an equal quantity of virus-containing suspension. In evaluating these results it should be considered that both vaccinated animals of the second experiment not only were inoculated with the virus but were exposed to kittens actually suffering from the infection.

The results in the vaccinated animals, none of which showed any indication of panleucopenia, while not conclusively demonstrating the value of the immunizing procedure, because of the relatively small number of animals involved—some of which, as we know from previous experience, might have possessed natural resistance—nevertheless strongly suggest that the vaccination did serve to prevent the appearance of any recognized signs of illness. In contrast, 2 of the 6 unvaccinated kittens died, and all exhibited definite indication of disease, although in one a significant leucopenia failed to appear.

Because of the frequency of spontaneous infections during the isolation-period of 2 weeks required for vaccination, we are able at this time to present only these 2 experiments. But something has been learned from many others of the same type which have been attempted. Thus they clearly showed that vaccination following exposure is ineffective, and that exposure soon after the beginning of vaccination results in a typical attack.

We have no knowledge of the duration of the immunity presumably induced by vaccination. Observations, however, on the persistence of the resistant state in animals which have recovered from infection suggest that a permanent immunity could be obtained by the inoculation of active virus following vaccination with formalinized material. A procedure of this sort apparently gives rise to humoral immune factors sufficient to protect kittens passively against a subsequent inoculation of active virus (see 4th experiment in Table II) and would be analogous to that which Laidlaw and Dunkin[5, 6]

[5] Laidlaw, P. P., and Dunkin, G. W., *J. Comp. Path. and Therap.*, 1927, **41**, 1.
[6] Laidlaw, P. P., and Dunkin, G. W., *Ibid.*, 1928, **41**, 209.

found successful in the immunization of dogs and ferrets against distemper.

Passive Immunization. Four separate experiments were carried out with the object of testing the prophylactic properties of convalescent and "hyperimmune" serum. Table II furnishes a summary of the significant facts. Kittens from 2 to 5 months of age were employed, and, in most instances, these were obtained in litters. The littermates were divided as equally as possible between the "serum-protected" and the control groups. This was done to equilibrate in so far as possible the immunologic status of the 2 groups. As in the experiments on active immunization, all kittens were obtained from country districts. Serum was given in one instance 33 days prior to the test-inoculation of virus and in the cases of 4 animals was not given until 3 days after adequate exposure. In addition to exposure this group of 8 kittens was also given virus by mouth at the time the serum was injected. Other kittens exposed at the same time as this group became infected without the additional administration of virus. In other experiments the serum was administered either at the same time as the virus or on the previous day.

The single death due to panleucopenia which occurred among the total 15 "serum-protected" animals was in one of 5 kittens which was not given serum until 3 days after exposure, or about the middle of the incubationary period, which in naturally acquired infections is from 6 to 9 days. One other death occurred in the serum-protected group in an animal suffering at the time of inoculation from a severe upper respiratory infection. At necropsy, including examination of tissue-sections, no evidence of viral infection could be found.

Only 2 of the 15 animals receiving serum developed a leucopenia: the fatal case mentioned above, and one other which had a count on one day of 8,150 leucocytes per cmm. This count falls just outside the lower limit of normal variation expected in this particular animal on the basis of statistical criteria established by Hammon[7] in a study of the physiological fluctuation of counts in a group of 66 kittens. All counts here recorded as leucopenic have been tested by these criteria.

The control kittens, which were equal in number to the serum-protected animals in each experiment, were given the same test-inoculation of virus at the same time, and kept in the same room and not infrequently in the same cage with the serum-protected animals. Eight of the 15 controls succumbed but only in 7 can death be attributed to infection with the virus, for one kitten died following

[7] Hammon, W. D., *Anat. Rec.*, March, 1939.

TABLE II.

Immunization-Experiments with Convalescent and Hyperimmune Serum.

	Serum inoculated							Controls without serum			
Exp.	Kitten No.	Type of serum	Dose, cc	When given	Mode of test infection	Leuco-penia	Out-come	Kitten No.	Mode of test-infection	Leuco-penia	Out-come
										3,900 (8)	
1	129	Convalescent (1)	2	33 days before test inoculation	Intraäbdominal	0	S	128	Intraäbdominal	+	S
		,, (1)	,,								
	133	Hyperimmune (2)	,,	15 ,, ,, ,, ,,	,,	0	S	131	,,	+	D
		Convalescent (1)	,,								
	134	Hyperimmune (2)	,,	15 ,, ,, ,, ,,	,,	0	D (6)	135	,,	+	D
										6,750 (8)	
					Repeated exposure				Repeated exposure		
2	169	Convalescent (3)	3	3 days after exposure and at	and *per os*	0	S	171	and *per os*	+	S
	170	,,	,,	same time as virus was	,,	0	S	173	,,	+	D
	172	,,	,,	given *per os*.	,,	0	S	174	,,	+	D (7)
	175	,,	,,		,,	0	S	176	,,	+	D
										400 (8)	
	177	,,	,,		,,	+	D	178	,,	+	S
										1,200 (8)	
3	200	,, (4)	4	1 day before test-inoculation	Intraäbdominal	0	S	203	Intraäbdominal	+	S
						8,150 (8)					
	201	,,	,,	,, ,, ,, ,, ,,	,,	+	S	204	,,	+	D
	202	,,	,,	,, ,, ,, ,, ,,	,,	0	S	205	,,	+	D
										6,900 (8)	
4	244	Hyperimmune (5)	4	Same time as ,, ,,	,,	0	S	240	,,	+	S
	245	,,	,,	,, ,, ,, ,, ,,	,,	0	S	241	,,	+	D
										4,550 (8)	
	246	,,	,,	,, ,, ,, ,, ,,	,,	0	S	242	,,	+	S
										6,050 (8)	
	247	,,	,,	,, ,, ,, ,, ,,	,,	0	S	243	,,	+	S
							2 D				8 D
Total	15					2	13 S	15		15	7 S

(1) Details of source uncertain.
(2) 10 months following recovery the cat from which the serum was obtained was reinoculated with virus, found resistant, and bled 17 days later.
(3) Bled 13 days after recovery.
(4) Bled 12 days after recovery.
(5) Bled 12 days after test-inoculation of virus following vaccination with formalinized tissue-suspension (cat 236, Table I).
(6) Had respiratory infection with temperature 103.4°F. when inoculated and died 7 days later with pneumonia. Necropsy showed no evidence of panleucopenia. Leucocyte-count at death was 68,800.
(7) During recovery (11 days after inoculation) pneumonia developed and was cause of death. Leucocyte-count had returned to normal.
(8) Lowest leucocyte-count obtained—counts made daily.

pneumonia several days after the leucocyte-count had become normal. All the unprotected kittens, however, developed a significant leucopenia (6,900 cells per cmm or less).

If the animals dying with pneumonia are eliminated from both protected and control groups, the results, on the basis of death or recovery when evaluated by statistical tests, are significantly different (χ^2 with the Yates correction for small numbers[8] = 4.37. P lies between .05 and .02). When tested for significance on the basis of infection as indicated by leucopenia the results become highly significant.

That convalescent serum given shortly before the appearance of symptoms fails to modify the disease is apparent from the following observation. Three kittens were given 4 cc of hyperimmune serum intraäbdominally 1 or 2 days before noticeable illness appeared. The ensuing disease ran a course similar to that occurring among the 3 control animals included in the experiment, although 2 cats died in the latter group and only one among those treated with serum.

Natural Immunity. An observation has been made which strongly suggests that the resistance of a naturally immune female cat is transferred to the offspring. During the early phase of our work in the winter of 1937-1938 we assembled a number of pregnant animals and mothers with suckling kittens, all of which were obtained in the vicinity of Boston, where the disease is endemic. Precautions were then taken to eliminate, in so far as possible, the introduction of infection among this group of about 30 cats. These proved eventually ineffective for after all the pregnant animals had given birth to their litters and at a time when the young ranged in age from 2 weeks to 2 months, one adult female and her 2 kittens died of panleucopenia. Heavy exposure of all other animals to the virus seems to have been assured, since they were loose in a common enclosure, the kittens appeared to suckle indiscriminately at times, and all attempts at further isolation were abandoned. Nevertheless, no other apparent infections occurred during the next few months, nor did the inoculation of any of these animals with organ-suspensions from infected cats lead to overt illness. This immunity of the kittens appears to be most easily accounted for *a priori* by a passive transfer of protective substance across the placenta. The duration of this type of immunity under effective isolation would at best be probably only a few months, but it would seem likely that when frequent exposure to the virus occurs, active immunity might fortify a waning passive

[8] Hill, Bradford A., *Principles of Medical Statistics*, The Lancet, Ltd., London, 1937, p. 93.

immunity. A chain of events of this sort could account for the fact, which we have noted, that relatively few manifest cases of this disease are seen in a community where it is endemic.

Comment. The evidence for the prophylactic action of convalescent or "hyperimmune" serum, provided this is given before or within 3 days after exposure, is, we believe, sufficient to justify its use to confer temporary protection upon cats before or after known contact with the disease. This information should be of value to those who employ cats for experimental purposes.

Should our observations on the immunizing properties of formalinized virus be supported by further experimental trials, the method would appear to offer a practical mode of inducing active immunity which, it is suggested, might be fortified and rendered more permanent by a subsequent inoculation of active virus. The use of the vaccine, however, would seem to be limited to animals which have not been exposed or which do not come in contact with the virus at least during the earlier period of time required for the procedure of vaccination.

Throughout our work we have employed a number of "strains" of virus obtained from animals derived from areas of the northeastern United States. These all produced the same clinical picture and the same pathological changes. Nevertheless, we have borne in mind the possibility that antigenic differences might distinguish strains of virus of different origin. The experiments reported here afford some evidence, however, which points to an antigenic homogeneity. Thus in the second experiment on active immunization and the fourth on passive protection, the viruses used in the production of vaccine or antiserum were obtained from sources other than those from which the viruses used for testing the immunity were secured. Apparently complete cross-protection occurred in both instances.

Summary. Evidence is presented which suggests that the injection of formalinized suspensions of organs from cats infected with the virus of malignant panleucopenia induces resistance in susceptible animals against a subsequent injection of active virus. The serum of cats convalescent from infection or that of animals vaccinated with formalinized material and then inoculated wth living virus, will protect susceptible cats from a subsequent or recent exposure to an animal suffering from the disease or from a subsequent injection of active virus.

11142 P

Influence of Sulfapyridine Therapy on Plasma Lipids in Pneumonia.*

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In the preceding paper[1] evidence has been presented to indicate that specific serum therapy is able to prevent the rather marked changes in the serum lipids which occur in pneumonia. The introduction of chemotherapy with its satisfactory clinical response brings up the question as to whether the more or less specific action of sulfapyridine in pneumococcal pneumonia has the same influence on the plasma lipids. Serum therapy and drug therapy both cause a rather prompt drop in temperature so that the patient is usually free of fever in 12 to 48 hours. However investigators have felt that the normal temperature which follows the administration of sulfapyridine does not necessarily indicate a complete recovery from the pneumonia. Just how the plasma lipids behave in cases receiving chemotherapy should therefore be of special interest.

This paper deals with a study including 5 children with pneumonia. The patients were between 6 and 9 years of age, all ill with lobar pneumonia due to the pneumococcus, Type I. They received sulfapyridine and since there were no hard and fast rules with regard to the amount of drug to be employed the dosage schedule recommended by Barnett and his co-workers[2] was at that time considered to be most satisfactory. The sulfapyridine was administered orally, 0.6 g being given every 4 hours throughout the 24 hours of a day. This group of selected patients did not have any vomiting prohibiting the retention of the drug. The first sample of blood was collected just before the sulfapyridine administration was started. Each child then had been ill for only one or 2 days and the fever was high, ranging between 40.2° and 40.8°C. The second sample was drawn about 24 hours after the temperature had dropped to normal. The third and fourth blood samples were obtained on the fourth and seventh days of convalescence, respectively. The drug was discontinued on the fourth day of normal temperature just after the third

* This work was supported by a grant from the Mead Johnson and Company and the Medical Research Fund of the University of Minnesota.

1 Stoesser, A. V., Proc. Soc. Exp. Biol. and Med., 1940, **43**, 168.

2 Barnett, H. L., Hartmann, A. F., Perley, A. M., and Ruhoff, M. B., *J. A. M. A.*, 1939, **112**, 518.

sample had been collected. As in the previous communication,[1] the subjects of this study received a thorough examination including a roentgenogram of the lungs, each time a sample of blood was drawn. Bloor's methods[3, 4, 5] were followed to determine the various cholesterol fractions. The total fatty acid and phospholipid values were obtained by the microgravimetric method of Wilson and Hansen.[6,7] Yasuda's modification[8] was employed to determine the iodine absorption of the serum fatty acids.

The results are summarized in Table I.

The infections in the 5 subjects resembled each other fairly closely. The lobar pneumonia was confined to the lower lobes of the lungs. The total cholesterol and esters were significantly lower than normal on the day the sulfapyridine was started. The response to this form of therapy was clinically quite satisfactory. In cases 2 and

TABLE I.
Plasma Lipids of Pneumonia Before, During, and After Administration of Sulfapyridine.

Case No.	Total cholesterol				Cholesterol esters				Free cholesterol			
	Mg per 100 cc serum											
	A	B	C	D	A	B	C	D	A	B	C	D
1. M.L.	126	135	124	238	85	76	73	160	41	59	51	78
2. G.M.	131	109	153	263	78	52	95	178	53	57	58	85
3. J.V.	101	110	122	214	43	55	71	137	58	55	51	77
4. E.T.	131	138	165	209	83	75	105	146	48	63	60	63
5. B.S.	142	107	121	227	94	68	69	169	58	39	52	58
Avg	126	119	137	230	76	65	82	158	51	54	54	72

	Total fatty acids				Iodine number				Phospholipids				Iodine number*			
	Mg per 100 cc serum															
	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
1. M.L.	360	280	435	514	93	88	88	95	103	72	103	138	104	108	110	120
2. G.M.	279	259	172	403	97	90	95	98	71	94	93	115	129	126	121	110
3. J.V.	214	286	298	329	81	87	95	98	72	122	121	159	105	76	77	149
4. E.T.	303	284	306	466	89	88	96	87	71	121	67	112	93	95	104	99
5. B.S.	276	307	249	367	104	98	93	100	81	94	68	72	122	106	97	118
Avg	286	283	292	415	92	90	93	95	79	99	90	119	110	102	101	119

* Iodine number of the phospholipid fatty acids.
A—Blood sample collected before sulfapyridine was started.
B—Blood sample collected after 24 hours of normal temperature.
C—Blood sample collected on 4th day of convalescent period.
D—Blood sample collected on 7th day of convalescent period.

3 Bloor, W. R., *J. Biol. Chem.*, 1916, **24**, 227.

4 Bloor, W. R., and Knudson, Arthur, *J. Biol. Chem.*, 1916, **27**, 107.

5 Bloor, W. R., personal communication to the author.

6 Wilson, W. R., and Hansen, A. E., *J. Biol. Chem.*, 1936, **112**, 457.

7 Hansen, A. E., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 376.

8 Yasuda, M., *J. Biol. Chem.*, 1931-32, **94**, 401.

3 the temperature fell slowly to within the normal range over a period of 2 days while in the remaining 3 patients there was a precipitous drop to normal in 12 to 36 hours. After one day of no fever, the second blood sample was obtained and the cholesterol values were found to have changed very little. On the fourth day of convalescence when the third sample of blood was collected there was some indication of a slight rise in the cholesterol. The observation is most interesting in view of the fact that the roentgenograms showed some extension of the pneumonia at the time the second blood sample was drawn and only a small amount of resolution on the fourth day of the convalescent period. However, following this, the pneumonic process began to resolve rapidly and on the seventh day of convalesence the lungs were clear in cases 1, 3, and 4. The other 2 patients still had incomplete resolution. The total cholesterol and esters had returned to the normal values.

The total fatty acids and phospholipid values were lower than the average figure during the period of the illness before the administration of the sulfapyridine. Although the introduction of drug therapy was followed by a good clinical response, the total fatty acids were slow in rising to higher levels. There was a slight rise and then another drop in the phospholipids and recovery from the low values was retarded. The iodine numbers of the total fatty acids and phospholipid fatty acids were not markedly depressed during the height of the disease. They apparently had little time to fall to the low levels observed during the last stages of the febrile period when the disease is allowed to take its natural course.[9] The sulfapyridine treatment definitely shortened the period of fever in each instance.

The drop in temperature is considered to be due to the direct action of the drug. It is not the natural crisis of the pneumococcal pneumonia and Wood and Long[10] have shown that the type-specific antibodies usually do not appear in the blood of these patients until several days later at the time when the natural crisis of an untreated case of pneumonia occurs. This is also the time when the plasma lipids first show signs of returning to normal levels. The question therefore arises as to whether the changes in the serum lipids are related to processes of immunity. Further investigations are in progress.

9 Stoesser, A. V., PROC. SOC. EXP. BIOL. AND MED., 1937, **36**, 723.

10 Wood, W. B., and Long, P. H., *Ann. Int. Med.*, 1939, **13**, 612.

11143

Imidazole Buffer: Its Use in Blood Clotting Studies.*

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In making studies on blood clotting we found it desirable to control pH in the range 6-8. Diethylbarbituric acid buffers have been recommended,[1] but the effective range is rather high (pH 7-9) and, furthermore, their solubility is not great. Buffers utilizing other weak acids, or salts, such as acid phosphate, tend to precipitate calcium or to form undissociated compounds. To avoid these difficulties we directed our attention to a study of the buffering action of weak bases. It was soon found that satisfactory results could be obtained with imidazole (glyoxaline). Shortly after our tests were carried out, Kirby and Neuberger[2] published a physicochemical study of imidazole derivatives. Working with a very dilute buffer solution (0.01 M), they found the pK_o value of imidazole to be 6.95. From this value one can calculate with reasonable accuracy the pH of dilute (0.01 M) buffer mixtures of imidazole and HCl. However, for the higher concentrations which are in more general use, the calculated values become approximations. To supply data, convenient for use, we wish to describe the preparation of imidazole buffers. We have chosen the concentration of 0.05 M, as being one which is more nearly suitable for many problems in biological research.

Imidazole was synthesized from tartaric acid by the method of Fargher and Pyman.[3] The white crystalline needles were dried over P_2O_5 and were found to have a melting point of 89° (corr.) and a nitrogen content which was 99.6% of the theoretical value. A portion of the imidazole used subsequently was obtained elsewhere.‡ Hydrogen ion determinations on buffer mixtures (Table

* Aided by a grant from the John and Mary R. Markle Foundation.

† Graduate Assistant, 1938-39, Graduate College, State University of Iowa.

1 Ransmeier, J. C., and McLean, F. C., *Am. J. Physiol.*, 1938, **121**, 488.

2 Kirby, A. H. M., and Neuberger, A., *Biochem. J.*, 1938, **32**, 1146.

3 Fargher, R. G., and Pyman, F. L., *J. Chem. Soc.*, 1919, **115**, 217.

‡ Part of our supply of imidazole was obtained through the courtesy of Dr. C. S. Marvel, University of Illinois. The rest was obtained from Eastman Kodak Company, Rochester, N. Y. The Eastman product, which is now generally available, should be dried before use.

TABLE I.
Imidazole Buffer.*

pH	0.1 N HCl cc	pH	0.1 N HCl cc
6.20	42.9	7.20	18.6
6.40	39.8	7.40	13.6
6.60	35.5	7.60	9.3
6.80	30.4	7.80	6.0
7.00	24.3		

*To the amounts of 0.1 N HCl listed in the table are added 25 cc portions of 0.2 M imidazole, and the mixtures are diluted with water to 100 cc.

In our original experiments, we used slightly different quantities of HCl (round number quantities), with corresponding differences in values of pH. The data actually obtained were plotted on a large-scale curve, and the values given in the table were obtained by interpolation.

I) were made at 25° ± 0.05°, using a glass electrode and a saturated KCl-calomel electrode (Beckman pH meter). Standardization was effected with standard acetate buffer having a pH of 4.62 at 25°. The pH values of newly prepared mixtures check the values in Table I within less than 0.02 pH. Buffer mixtures which were allowed to stand for 2 months at room temperature showed no growth of microörganisms and no detectable change in pH. It is of interest to note that imidazole buffer covers a pH range which is almost identical to that covered by mixtures of primary and secondary phosphates.[4]

In blood clotting studies we use the various reagents in concentrations which are isosmotic with 0.9% NaCl. Imidazole buffer, pH 7.25, which is isosmotic with 0.9% NaCl, as determined by the freezing point method, is prepared by dissolving 1.72 g of imidazole in 90 cc of 0.1 N HCl, and diluting with water to 100 cc. For the past year, we have included this imidazole solution among the reagents used in the 2-stage technique for the titration of prothrombin.[5] Tests show that the buffer has no effect upon either the conversion of prothrombin into thrombin, or upon the reaction of thrombin with fibrinogen, even when the buffer solution comprises more than one-half of the total volume.

Summary. Mixtures of imidazole and hydrochloric acid covering the pH range 6.2 to 7.8 are described. These mixtures are recommended as buffers for reactions which require the presence of calcium ion.

4 Clark, W. M., and Lubs, H. A., *J. Biol. Chem.*, 1916, **25**, 479.

5 Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, **114**, 667; *J. Exp. Med.*, 1937, **66**, 801.

11144

Composition of Bone in Extreme Osteoporosis Associated with Hepatoma.

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The presence of a marked degree of decalcification of the entire skeleton in a 10-year-old boy suffering from extensive carcinomatous involvement of the liver prompted the investigation herein reported. A preliminary report of the clinical and laboratory studies in this case was given last year by Hansen, Ziegler and McQuarrie.[1] The osteoporosis developed early in the course of the disease and continued to progress throughout the 18 months he was under observation. The specimen of rib bone obtained shortly after exitus was freed from all muscular attachments, cleaned, weighed and stored at −20°C until ready for analysis. The entire specimen was prepared for analysis in each case. The manner of preparation of the specimens, the chemical methods, and system of calculations were the modification of Kramer and Howland's[2] procedure, and are reported elsewhere.[3] The results of the analyses, calculated on green, dry, and dry extracted bases are presented in Table I, together with similar data obtained on the rib bones of a 9-year-old boy having no demonstrable abnormality of the osseous system.

The data in Table I show that there had been a marked replacement of bone minerals by lipid and water, amounting to 27.8% when regarding the bones of the control subject as normal. Slightly more than 80% of this was lipid (alcohol-ether soluble) material. The data also show that the dry, lipid-free bone of the hepatoma patient had 31.4% less Ca, 33.8% less P, 39.0% less Mg and 37.9% less CO_2 than that of the control. On the basis of the system of calculation employed, it appears from these results that there was relatively more $CaCO_3$ and $Mg_3(PO_4)_2$ than $Ca_3(PO_4)_2$ withdrawn from the bone. The calculated ratio, residual Ca:residual P, is thus about 5% less in the abnormal bone. This difference, however, is probably not significant, for it is within the range of such

[1] Hansen, Arild E., Ziegler, M. R., and McQuarrie, I. *Proc. Am. Soc. Exp. Path., Arch. Path.*, 1938, **25**, 757.

[2] Kramer, B., and Howland, J., *J. Biol. Chem.*, 1926, **68**, 711.

[3] Neal, W. M., and Palmer, L. S., *J. Agr. Res.*, 1931, **42**, 107.

TABLE I.

Percentage Composition of Green, Dry, and Dry Extracted Bone from Child with Hepatoma (Case 1, L.C.) Together with Similar Data from Child with No Abnormality of the Osseous System (Case 2, G.L.).

	Case 1, L.C.	Case 2, G.L.			
	Rib	7th rib	8th rib	Mean	Difference*
H_2O	48.05	36.00	33.76	34.88	
Alcohol and ether extract { Green	16.32	3.68	3.24	3.46	
Alcohol and ether extract { Dry	31.42	5.75	4.89	5.32	
Ash { Green	14.87	35.56	35.72	35.64	58.2
Ash { Dry	28.63	55.56	53.93	54.75	47.8
Ash { " extracted	41.74	58.95	56.70	57.83	27.8
Ca { Green	5.08	13.75	14.15	13.95	
Ca { Dry	9.78	21.49	21.36	21.43	
Ca { " extracted	14.36	22.80	22.46	22.63	31.4
P { Green	2.41	6.24	6.34	6.29	
P { Dry	4.64	9.75	9.57	9.66	
P { " extracted	6.76	10.35	10.06	10.21	33.8
Mg { Green	0.067	0.196	0.182	0.189	
Mg { Dry	0.129	0.307	0.275	0.291	
Mg { " extracted	0.188	0.326	0.289	0.308	39.0
CO_2 { Green	0.75	2.06	2.11	2.09	
CO_2 { Dry	1.44	3.22	3.18	3.20	
CO_2 { " extracted	2.10	3.42	3.34	3.38	37.9
Ca as $CaCO_3$ (.91 × $\%CO_2$)	1.91	3.11	3.04	3.08	38.0
P as $Mg_3(PO_4)_2$ (.86 × %Mg)	0.16	0.28	0.25	0.27	40.7
Residual Ca (%Ca — Ca as $CaCO_3$)	12.35	19.69	19.42	19.56	36.9
Residual P (%P — P as $Mg_3(PO_4)_2$)	6.60	10.07	9.81	9.94	33.6
Ratio $\frac{\text{Residual Ca}}{\text{Residual P}}$	1.871	1.955	1.980	1.968	4.9
$CaCO_3$ (2.27 × $\%CO_2$)	4.77	7.77	7.59	7.68	37.9
$Ca_3(PO_4)_2$ (5 × %Residual P)	33.00	50.35	49.05	49.70	31.6
Ratio $\frac{Ca_3(PO_4)_2}{CaCO_3}$	6.918	6.480	6.462	6.471	+7.0
$Mg_3(PO_4)_2$ (3.6 × %Mg)	0.68	1.17	1.04	1.11	38.7
Total $CaCO_3 + Ca_3(PO_4)_2 + Mg_3(PO_4)_2$	38.45	59.29	57.68	58.49	34.3

*Percentage difference between normal specimen and specimen from hepatoma case.

values reported by Howland, Marriott and Kramer.[4] More significant is the calculated $Ca_3(PO_4)_2$:$CaCO_3$ ratio, which is definitely higher in the abnormal bone. It has been shown[4] that this ratio decreases in rickets and that it also decreases in phosphorus deficiency,[5] but rises in calcium deficiency. Therefore, it appears that the situation encountered in this bone abnormality is more analagous to calcium deficiency than to either phosphorus deficiency or rickets.

4 Howland, John, Marriott, W. McKim, and Kramer, Benjamin, *J. Biol. Chem.*, 1926, **68**, 721.

5 Neal, Wm., Palmer, L. S., Eckles, C. H., and Gullickson, T. W., *J. Agr. Res.*, 1931, **42**, 115.

11145 P

Potency Evaluations of the Human Chorionic Gonadotropic Preparations.

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There are 7 preparations of the gonadotropic principle of human pregnancy urine in common use at the present time. All of these are standardized in rat units. However, each manufacturer defines his unit differently, using various criteria as a basis.

Estrous type of vaginal smear, follicular maturation and luteinization, and change in ovarian weight are some of the criteria employed. The age of the test animals, the time elapsed during the assay, and the number of injections, are also variables.

Since different quantities of the human urinary gonadotropic material are required to produce these criteria, and since an older test animal is more easily brought to maturation than a younger one, the commercial human urinary gonadotropic units are not comparable by definition alone.

D'Amour and D'Amour[1] have attempted to make an effective comparison of these units, employing ovarian weight as a criterion. We have adopted the estrous type of smear in 21-day-old rats as the criterion in our assays. All our animals were females of the Sherman strain, and at least 10 rats were utilized for each assay. All available human urinary gonadotropic products were then assayed by this method.*

Daily injections of 0.5 cc of the test material were given subcutaneously for 3 consecutive days, and the animals were tested for the estrous type of smear at 120 hours following the initial dose. Their ovaries were examined grossly for evidences of maturation. A result was considered positive if at least 50% of the animals had an estrous type of smear.

If the number of rat units of the test substance first employed was insufficient to give a positive result, a greater number of units was injected into each of the next group of 10 rats. If the first test was positive, the number of rat units was reduced until the

[1] D'Amour, M. D., and D'Amour, F. E., *J. Pharm. and Exp. Therap.*, 1938, **62**, 263.

* All products were bought from a reputable pharmacist.

TABLE I.
Production of the Estrous Type of Vaginal Smear.

Preparation	Lot No.	Total Dose, R.U.	No. of rats used	No. of rats in estrus	% of rats in estrus	Relative potency
Antuitrin-S	3203307	3.0	9*	9	100	
"	"	2.25	10	8	80	
"	3220458	1.5	10	5	50	
"	"	1.0	10	1	10	1.00
A.P.L.	RB556	3.0	10	1	10	
"	"	4.0	10	4	40	
"	"	4.5	10	5	50	0.33
Entromone	10981	3.0	10	0	0	
"	"	6.0	10	0	0	
"	"	9.0	10	0	0	
"	"	12.0	10	0	0	0.00†
Follutein	65467	1.5	10	6	60	
"	"	1.0	10	1	10	1.00
Korotron	CN415	1.0	10	1	10	
"	"	1.5	10	3	30	
"	"	2.0	10	0	0	
"	"	2.5	10	1	10	
"	"	3.0	10	9	90	
"	8204	1.0	10	0	0	
"	"	1.5	10	0	0	
"	"	2.0	10	0	0	
"	"	2.5	10	2	20	
"	"	3.0	10	1	10	
"	"	4.0	10	3	30	
"	8900	5.0	10	10	100	?‡
Placestrin	27	1.5	10	0	0	
"	"	3.0	10	0	0	
"	"	6.0	10	0	0	
"	"	7.5	10	0	0	
"	"	10.0	10	0	0	0.00†
Pregnyl	§	3.0	10	7	70	
"	"	1.5	10	7	70	
"	"	1.0	10	3	30	1.00

*One of the 10 rats died in this assay.
†These products were found to be impotent in the dosage employed.
‡The marked variations in potency of individual ampules make it impossible to rate this product.
§Lot number not noted.

minimum amount necessary to produce a positive result was ascertained. In all doubtful cases the experiment was repeated.

All these preparations were tested for the presence of estrin which would invalidate results based on an estrous type of smear. None of the assayed products contained any estrogenic material.

These results suggest that the potencies of the human urinary gonadotropic rat units employed by the manufacturers vary greatly. To remedy this situation a uniform standard should be adopted.† This has already been accomplished with the estrogenic preparations.

11146

Protecting Action of Procaine Against Ventricular Fibrillation Induced by Epinephrine During Cyclopropane Anesthesia.

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There is much evidence indicating that several anesthetic agents sensitize the heart so that the addition of small amounts of epinephrine may cause ventricular fibrillation. Oliver and Schafer[1] were first to observe this as a frequent complication during chloroform anesthesia, and Levy[2] elaborated on the reaction. More recently, Meek, Hathaway and Orth[3] have shown that the same effect may be produced in the dog during cyclopropane anesthesia. Experimental data accumulated by Kochman and Daels,[4] Mautz,[5] and Beck and Mautz[6] serves to establish that procaine applied locally to the heart reduces irritability of the myocardium as evidenced by augmentation in intensity of stimulation necessary to produce extra-systoles or ventricular fibrillation. Hermann and Jourdan[7] have reported that following subcutaneous injections of procaine a larger dose of epinephrine is necessary to produce ventricular fibrillation during chloro-

† Since the completion of these experiments, the League of Nations Health Organization has set up an international unit for the human chorionic gonadotropin. It is designated as 0.1 mg of a standard preparation of a gonadotropic extract from human pregnancy urine. This unit is supposed to produce an estrous type of smear in 21-day-old rats.

1 Oliver, G., and Schafer, E. A., *J. Physiol.*, 1895, **18**, 230.

2 Levy, A. G., *J. Physiol.*, 1911, **43**, 3.

3 Meek, W. J., Hathaway, H. R., and Orth, O. S., *J. Pharm. and Exp. Therap.*, 1939, **61**, 240.

4 Kochman, M., and Daels, F., *Arch. Internat. Pharm.*, 1908, **18**, 41.

5 Mautz, F. R., *J. Thoracic Surg.*, 1936, **5**, 612.

6 Beck, C. S., and Mautz, F. R., *Ann. Surg.*, 1937, **106**, 525.

7 Hermann, H., and Jourdan, F., *C. R. Soc. de Biol.*, 1931, **106**, 1153.

form anesthesia. Shen and Simon[8] have shown that procaine, given to dogs simultaneously with epinephrine during chloroform anesthesia, protects against fibrillation. The present study was undertaken to determine whether similar results could be obtained during cyclopropane anesthesia.

Thirty-eight experiments were performed on fourteen dogs. Preanesthetic medication, morphine sulphate one mg per kilo and scopolamine hydrobromide 0.04 mg per kilo was injected subcutaneously one hour before each experiment. The carbon dioxide absorption technic was utilized for cyclopropane anesthesia. An unobstructed airway was assured by an endotracheal tube fitted with an inflatable cuff. Depth of anesthesia was maintained at second plane as evidenced by the loss of the lid reflex and maintenance of intercostal activity. Electrocardiograms (lead II) were taken before, during and after drug administration.

The test injection of epinephrine was 0.01 mg per kilo in 5 cc of normal saline, given intravenously at the rate of 1 cc per 10 seconds. The test injection of procaine was 5 mg per kilo in 5 cc of normal saline, given intravenously, 1 cc in 10 seconds. Electrocardiographic records were completed during cyclopropane anesthesia using epinephrine alone, procaine and epinephrine simultaneously, procaine preceding epinephrine and epinephrine preceding procaine.

The effects of injecting epinephrine alone were studied in 5 dogs. Three of the animals died of ventricular fibrillation. Two of these had recovered from previous experiments in which they had been treated with procaine prior to the intravenous injection of the test dose of epinephrine. Following an interval of several days these animals were given an identical dose of epinephrine alone and both developed ventricular fibrillation. Of the other 3 dogs 2 developed ventricular tachycardia but recovered; the third developed ventricular tachycardia, then fatal ventricular fibrillation.

Procaine and epinephrine were given simultaneously to 5 dogs. Two died of ventricular fibrillation. The 3 surviving animals developed electrocardiographic phenomena referable to the A-V node and the ventricles which rapidly shifted back to the sinus node. All revealed increased cardiac rates, which suggested an initial rapid and predominant epinephrine effect with gradual and slower ascendency of the procaine effect and reëstablishment of the pacemaking sinus node.

Procaine before epinephrine was given to 8 dogs, 7 recovered. One

8 Shen, T. C. R., and Simon, A., *Arch. Internat. de Pharm. et Therap.*, 1938, **59**, 68.

of these animals died when an amount equal to 8 test doses of epinephrine was injected intravenously. This same dog had been studied twice before this fatal termination by injecting procaine before epinephrine, employing one test dose of epinephrine the first time and 3 test doses the second time, when the only electrocardiographic abnormality was short runs of ventricular tachycardia.

Epinephrine was given before procaine in 8 experiments with 5 dogs. The same experiment was repeated in 2 of the animals. All the animals survived and the results did not vary; that is, after ventricular tachycardia was established following the intravenous injection of a test dose of epinephrine, the intravenous or intracardiac injection of a test dose of procaine caused a change to auricular tachycardia which then reverted to a sinus rhythm.

Conclusions. The experiments presented support the contention that when procaine is administered to cyclopropanized dogs the incidence of ventricular fibrillation following epinephrine administration is reduced. Sixty percent of the animals developed ventricular fibrillation after injection of one test dose of epinephrine. When procaine was previously administered all animals were protected, and the introduction of 8 test doses of epinephrine were required to produce the fatal complication. The results also indicate that procaine may be efficient in the treatment of ventricular fibrillation induced in the dog by epinephrine during cyclopropane anesthesia. In every instance in which procaine was given intravenously at the time when ventricular tachycardia occurred following the administration of epinephrine, recovery was effected.

11147 P

Estrogenic Therapy by Implantation of Stilbestrol Pellets.

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The new synthetic estrogen, diethylstilbestrol (4:4-dihydroxy-alpha:beta-diethyl stilbene), announced by Dodds and coworkers,[1] has been found capable of producing in animals all the effects previously obtained with naturally occurring estrogens. Early reports

[1] Dodds, E. C., Lawson, W., and Noble, R. L., *Lancet*, 1938, **1**, 1389.

indicated that it would be clinically useful when we began studies upon patients in March, 1939. We found in 41 patients (14 complete castrates, 7 partial castrates, 5 patients with primary eunuchoidism and amenorrhea, and 15 patients with severe symptoms of the spontaneous menopause), that relief of the nervous and vasomotor symptoms, estrous changes in the vaginal smears, proliferative endometrial changes and breast growth could be produced by oral or subcutaneous administration of 1 mg to 5 mg daily of diethylstilbestrol dipropionate. An analysis of these studies is to be published elsewhere.

To determine whether subcutaneous pellet implantation would be effective, hard pellets of diethylstilbestrol dipropionate weighing approximately 100 mg each were implanted in 6 women who had had both ovaries removed. The sterile pellets were inserted through a small subcutaneous incision in the lumbar region and the wound closed with a stitch. Within 2 days the castrate type vaginal smears showed estrous changes and within 7 to 10 days this response was marked. Endometrial biopsies showed active proliferation within 7 days. The menopausal symptoms, both psychic and vasomotor, were greatly relieved. These changes persisted as long as the pellets remained in place. Symptoms recurred within about 2 weeks in 3 cases when pellets were removed and not replaced.

The pellets were extracted and weighed at intervals and the average daily absorption calculated by the weight loss. Pellets have been removed after periods varying from 27 to 53 days. Average daily absorption per pellet has ranged from 0.127 to 0.250 mg, corresponding to a dose of 2,540 to 5,000 international (estrone) units daily. One patient has been maintained on pellet therapy alone for over 4 months, and 3 others for over 2 months each, all with persistent improvement. There is practically no local reaction to the pellets. They become inclosed in a tight fibrous capsule which must usually be removed with the pellet if the pellet is to be obtained intact.

When the dose necessary to produce satisfactory relief of symptoms and active estrous effects upon the vaginal smears and endometrium is compared, it is found that a fraction of the oral or injection dose is sufficient by subcutaneous pellet implantation. Stilbestrol administered by pellet implantation seems to be from 5 to 10 times as effective per unit weight.

The method offers an effective and economical mode of estrogen therapy. One such small "artificial ovary" should furnish sufficient hormone in the average menopausal or oöphorectomized patient to last approximately 400 to 800 days. A larger dose can be furnished

by inserting more than one pellet through a single incision. Smaller doses no doubt would be obtainable by using smaller pellets. No untoward symptoms or toxic effects were noted. Repeated tests of liver function, red blood cell, hemoglobin, white blood cell and blood platelet determination showed no variation from normal.

11148

Peptone-Dextrose Broth for Use in Studies of Antibacterial Activity.*

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A peptone-dextrose broth for use in measurements of the bactericidal activity of sulfanilamide and sulfapyridine has been described.[1, 2] This broth (PD) consisted of 0.7% Neopeptone (Difco), 0.7% Proteose peptone (Difco), and 0.7% Pfanstiehl peptones plus 0.1% dextrose and 0.5% sodium chloride buffered at pH 7.5 ± 0.1. Bactericidal action with the two compounds mentioned above, in concentrations of 10 mg % or less, was demonstrable only at incubation temperatures above 37°C. This bactericidal action was accompanied by abundant control growth in bacteria-broth mixtures containing no drug. The test culture used in these studies was Beta hemolytic streptococcus strain C 203.

Recent attempts to utilize PD broth, made from currently available batches of peptones, in continued studies of antibacterial activity, have been unsuccessful due to failure to obtain growth in broth control tubes at the elevated incubation temperatures which are required for demonstration of bactericidal action with sulfonamide type compounds. Modification of the peptone dextrose test medium thus became necessary for reproduction of previously reported results and for continuance of these studies.

Preliminary tests indicated that a pH of 7.2 ± 0.1 was optimal for growth of strain C 203 in peptone-dextrose broth. However, broth buffered at pH 7.2, and containing peptones as described above,

* This investigation has been aided by a grant from the John and Mary R. Markle Foundation.

1 White, H. J., and Parker, J. M., *J. Bact.*, 1938, **36**, 481.

2 White, H. J., *J. Bact.*, 1939, **38**, 549.

failed to support growth at 39°C even when the dextrose content was increased to 0.2%. Tests to determine the growth-supporting ability of each of the 3 peptones were then carried out. Tryptose peptone (Difco) was also included in these tests. Broths, buffered at pH 7.2, containing 0.2% dextrose and 0.5% sodium chloride, but with different combinations of the peptones, were compared in terms of their ability to support growth of an initial concentration of approximately 5000 units of strain C 203 (as determined by plate counts). Of the 4 brands of peptone tested, only Tryptose and Pfanstiehl appeared to be suitable for the growth of strain C 203 at 37°C. Neither of these 2 peptones alone supported growth at 39°C. Broths which supported growth at elevated temperatures were obtained when a concentration of 2.0% Tryptose combined with Pfanstiehl in a concentration of from 0.06% to 0.24% was used. Mixtures of 1.0% Tryptose and 1.0% Pfanstiehl, 2.0% Pfanstiehl and 0.06% Tryptose, and certain other combinations of these 2 peptones, failed to support growth at 39°C.

On the basis of these results, our peptone-dextrose medium for antibacterial activity tests was modified as follows:

Peptone-Dextrose Broth (Modified).

Distilled water	1000 cc
Tryptose peptone (Difco)	20.0 g
Pfanstiehl peptone	1.0 g
Sodium chloride	5.0 g
Phosphate buffer†	40.0 cc

Heat ingredients listed above for 10 minutes at 100° C. Add 2.0 g of dextrose, dispense and autoclave for 20 minutes at 112° C to sterilize. Final pH 7.2 ± 0.1.

This modified broth has supported growth of small inoculums of strain C 203 at temperatures above 39°C. Reproduction of the

TABLE I.

Bactericidal Activity of Sulfanilamide Against Strain C 203 at Incubation Temperatures Between 37 and 39.5°C.

Initial Bacterial Concentration	Minimal Bactericidal Concentration of Sulfanilamide in mg%			
	37°C	38°C	39°C	39.5°C
5,000,000	800	600	400	200
500,000	600	600	200	60
50,000	600	400	80	8
5,000	400	200	40	4
500	200	60	8	2
50	80	20	6	1

Initial bacterial concentrations listed above indicate the average number of bacterial units per cc of test mixture, as determined by plate counts. Minimal bactericidal concentrations of sulfanilamide represent the lowest concentrations of drug required to obtain sterilization of test mixtures during 48 hours' incubation.

† Phosphate buffer mixture containing 0.4 M $Na_2 HPO_4 \cdot 12 H_2O$ and 0.1 M $NaH_2 PO_4 \cdot H_2O$ (pH 7.2).

previously determined[2] bactericidal activity of sulfanilamide and sulfapyridine has been obtained in this broth. Its use in measurements of the *in vitro* activity of other sulfonamide-type compounds will be subsequently reported.

Table I contains data which are typical of the results obtained when the modified broth is used as a test medium in studies of the bactericidal activity of sulfanilamide. Our test procedure and criteria of bactericidal activity have been described elsewhere.[2] From this table it is evident that previous observations of a striking increase in the bactericidal power of sulfanilamide, coincident with a temperature change from 37° to slightly above 39°C., are confirmed by the present data.

Summary. (1) Peptone-Dextrose broth (PD), made with currently available samples of Neo-, Proteose and Pfanstiehl peptones, has failed to support growth of beta hemolytic streptococcus strain C 203 at the elevated incubation temperatures which are required for demonstration of bactericidal activity with low concentrations of sulfanilamide. (2) A modified PD broth containing 2.0% Tryptose and 0.1% Pfanstiehl peptones together with 0.2% dextrose and 0.5% sodium chloride buffered at pH 7.2 has been found to be satisfactory for use in studies of bactericidal activity. (3) Results obtained with this modified broth confirmed the conclusions previously drawn in regard to the critical relationship between temperature and the bactericidal activity of sulfanilamide.

11149

Androgenic Effects from Percutaneous Administration in Castrate Rats.*

BIRDIE L. SCOTT. (Introduced by Carl R. Moore.)

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It has been demonstrated[1] that androgens in lanolin ointments are readily absorbed when applied on the skin. The work revealed that testosterone and t-propionate produce, when absorbed through the skin, measurable effects such as those known to result from subcutaneous injections of these hormones in oil. It was pointed out that

* This investigation has been aided by a grant from the Rockefeller Foundation to the University of Chicago.

[1] Moore, Carl R., Lamar, Jule K., and Beck, Naomi, *J. A. M. A.*, 1938, **111**, 11.

the superiority of the propionate, demonstrable when injected subcutaneously in oil, was lost in percutaneous administration.

An extension of these studies has appeared advisable and the observations reported herein compare the relative effectiveness of percutaneous applications of testosterone, t-propionate, and methyltestosterone when each is carried in a lanolin and in a tegin base.†

Experimental Procedure. Castrated male rats of different ages constituted 4 experimental groups. Treatment was given by daily applications of the ointment on an area of the neck and shoulders shaved cleanly of hair. Adult males were castrated 21 or 22 days prior to first treatment and young males were castrated 30 days after birth and received first treatment 12 days later. A chosen receptacle, adaptable as an accurate measure, was selected for each group and the average weight of 7 full receptacles of the base containing hormones constituted the daily dose. Animals were so caged as to avoid any contamination by contact with untreated, or differently treated ones. Twenty daily treatments were followed one day later by careful autopsy involving dissection under a binocular dissecting microscope of the ventral prostate gland, coagulating glands, seminal vesicles and periurethral tissue along with the dorsal prostate (for anatomy and terminology see figure 26, Moore '39[2]); tissues were weighed fresh on a torsion balance. Average weights of ventral prostates and seminal vesicles are recorded in the table to demonstrate the magnitude of hormonal responses.

Results. A histological study of the ventral prostate gland and seminal vesicles from this series of treated castrates reveals that in all cases the dosage employed was sufficient to maintain the normal histological secretory condition of these accessory reproductive organs, hence relative effects of the different preparations must be reflected in the weight responses of the organs in question. Ventral prostates revealed well defined light areas in the secretory cells,[3] and secretory cells of the seminal vesicles contained well defined secretion granules.[4] With responses of this intensity the histological conditions are not adequate to determine relative effects but weights of fresh tissues adequately demonstrate quantitative responses.

† These hormones in different cream bases have been graciously prepared by Dr. Erwin Schwenk of Schering Corporation; concentrations were 2 mg of hormone per gram of cream.

2 Moore, Carl R., *Sex and Internal Secretions,* Williams and Wilkins, Baltimore, 1939, Chapter 7.

3 Moore, Carl R., Price, Dorothy, and Gallagher, T. F., *Am. J. Anat.,* 1930, **45**, 71.

4 Moore, Carl R., Hughes, Winifred, and Gallagher, T. F., *Am. J. Anat.,* 1930, **45**, 109.

TABLE I.
Effect of Androgen-containing Ointments on Prostate and Seminal Vesicles of Castrate Rats.

	No. of animals	Body wt, g	Ventral prostate, mg: Actual	Ventral prostate, mg: Per 100 g body wt	Seminal vesicles, mg: Actual	Seminal vesicles, mg: Per 100 g body wt
A. Castrated on day 30; 1st treatment 12 days later; 20 daily treatments with 102 mg ointment, containing 0.20 mg hormone.						
Castrated controls	2	167	6.9	4.1	6.5	3.8
Blank tegin	2	142	7.2	5.0	5.6	3.9
Tegin—testosterone	2	170	74.3	43.7	80.4	47.2
" —t-propionate	2	160	57.9	36.1	53.0	33.1
Blank lanol	2	150	8.1	5.4	6.6	4.4
Lanol—testosterone	2	180	70.8	39.3	49.8	27.6
" —t-propionate	2	157	31.0	19.7	19.4	12.3
B. 4½-month males, castrated 21 days prior to treatment. 20 daily treatments with 165.2 mg ointment, containing 0.33 mg hormone.						
Blank tegin	2	217	30.0	13.8	82.6	38.0
Tegin—testosterone	2	295	275.7	93.4	425.5	144.2
" —t-propionate	2	245	182.3	74.4	407.8	166.4
Blank lanol	2	255	31.4	12.3	89.5	35.0
Lanol—testosterone	2	280	203.7	72.7	449.9	160.6
" —t-propionate	2	305	102.8	33.7	111.4	36.5
C. 3½-month males, castrated 22 days prior to treatment. 20 daily treatments with 101.6 mg ointment, containing 0.203 mg hormone.						
Castrate control	1	268	26.4	9.8	89.6	33.4
Tegin—testosterone	2	299	185.2	61.9	404.3	135.2
" —methyl-test.	2	264	251.3	95.1	762.1	288.6
Lanol—testosterone	2	279	140.0	50.1	333.7	119.6
" —methyl-test.	2	257	212.4	82.6	357.0	138.9
D. 4½-month males, castrated 22 days prior to treatment. 20 daily treatments with 179.8 mg ointment, containing 0.359 mg hormone.						
Castrated controls	3	334	46.6	13.9	100.0	29.9
Tegin—testosterone	3	277	367.8	132.7	701.2	253.1
" —methyl-test.	3	266	299.0	112.4	712.7	267.9
Lanol—testosterone	3	281	249.1	88.6	478.2	170.1
" —methyl-test.	3	302	283.9	94.0	506.7	167.7

Table I reveals the actual fresh weight of ventral prostates and seminal vesicles as averages from similarly treated males, as well as the weight of these two organs per 100 g of rat.

Close attention to this table reveals several points of interest: (1) In Group A the tegin and lanol bases alone, applied daily to castrate rats as controls, failed to effect appreciably the weight of these organs, hence responses to the same bases carrying hormones clearly demonstrate the effect of the hormone. (2) Comparisons of equal weights of crystalline testosterone with t-propionate (A series 0.2 mg; B series 0.33 mg) reveal the superiority of free testosterone whether the substances are carried in a tegin or lanol base; the one exception occurs in series B in which seminal vesicle response, computed as per 100 g of rat, gives a slightly higher value for t-propionate administered in the tegin base. (3) Methyl-testosterone applications

in comparison with applications of free testosterone (C series 0.203 mg; D series 0.36 mg) produced slightly higher end organ weights in 13 cases and slightly lower weights in 3 cases. Whereas the differences are not profound in series D the trend in all compared treatments employing these 2 hormones suggests a slight advantage for methyl-testosterone. (4) A comparison of effects of the same hormone (testosterone, t-propionate, m-testosterone) carried in the 2 different bases, and in all different dosages employed, suggests that tegin provides a more effective base than lanol. It is apparent from the table that end organ weights were greater after application of hormones in the tegin base in 30 comparisons as against 2 comparisons in which hormone in the lanol base produced greater weights (Ser. B comparing free test on sem. ves. weight).

Conclusions. Based upon weight responses evoked in the end organs of castrated male rats, the results obtained with androgens, percutaneously administered, indicate the effectiveness to follow the descending order—methyl-testosterone, free testosterone and t-propionate (lowest), although the differences between the first two substances are not great. It is further suggested that androgens carried in a tegin base are more effective than when carried in a lanol base, since end organ weights were greater in 30 of the 32 compared pairs. With all dosages the histologically indicated secretory normality was apparent for both ventral prostates and seminal vesicles.

I wish to acknowledge grateful appreciation to Dr. Carl R. Moore for directing this investigation, to Dr. Dorothy Price for friendly interest and assistance, and to Mrs. Dorothy Plagge for aid with operations.

11150

Pathways of Enzymes into the Blood in Acute Damage of the Pancreas.*

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It has been known for a long time that in acute diseases of the pancreas and in injury of the pancreas, the amylase concentration in the blood increases acutely. To our knowledge, the pathway by which pancreatic enzymes get into the blood stream has not been analyzed. In order to do this, experiments were performed on dogs under pentobarbital sodium anesthesia. A peripheral vein was prepared for withdrawal of blood and in a number of animals the thoracic duct was cannulated in the neck and the lymph collected. The pancreas was exposed and the main pancreatic duct prepared. In 9 experiments, dog's gallbladder bile in amounts varying from 0.1 to 4 cc was then injected into the duct after which the duct was ligated; several samples of portal blood were withdrawn in some of these experiments. In one experiment the pancreatic duct was ligated only; no bile was injected. In this and in one other experiment in which the pancreatic duct was not ligated and in which the pancreas was not touched at all, secretin was given intravenously in order to stimulate the external secretion of the pancreas.† Venous blood and thoracic duct lymph were collected at regular intervals before and after the above procedures. In these samples amylase was determined using the method of Wohlgemuth[1]‡ and lipase, using the method of Crandall and Cherry.[2] Trypsin determinations were performed in some instances but were of no value.

Results. Because our results were quite uniform, only a few representative experiments are given in Table I. It is apparent that in the fasting anesthetized dog, the concentrations of amylase in peripheral and portal blood and in thoracic duct lymph are almost equal. A rise in amylase concentration of the blood may appear as early as 5-10

* Aided by the A. B. Kuppenheimer Fund.

1 Wohlgemuth, J., *Bioch. Z.*, 1908, **9**, 1.

† Secretin Astra was used for which we are much obliged to Prof. Hammarsten, Dr. Agren and Astra Company, Sweden.

‡ Amylase concentration is expressed by the degree of dilution of 1 cc of serum at which 1 cc of the diluted serum will digest 1 cc of a 0.1% starch solution at 38°C within 30 minutes.

2 Crandall, L. A., Jr., and Cherry, I. S., *Am. J. Physiol.*, 1932, **100**, 266.

TABLE I.
Amylase and Lipase in Blood and Lymph.

Exp. No.	Procedure	Time after procedure, min.	Peripheral blood		Portal blood		Lymph (thoracic duct)	
			Amylase	Lipase	Amylase	Lipase	Amylase	Lipase
1.	4.0 cc bile inj.	0	64				64	
		5	64		128		64	
		15	128		128		128	
		60	128		128		512	
		120	128		128		512	
2.	0.4 " " "	0	32				32	0.16
		5	128					
		10	128				512	0.82
		30	128				512	
		60	256				2048	1.87
3.	2.0 " " "	0	64	0.20			32	0.06
	Occlusion of	10	64	0.18	256	0.93	64	
	portal vein for	30	512				1024	
	10 minutes	60	512	1.18			2048	1.65
4.	Ligature of	0	32				32	
	main pan-	10	64				32	
	creatic duct	30	64				64	
	Secretin i.v.	10	64				1024	
	200 cat units	30	64				1024	
		60	256				1024	
5.	Secretin only	0	64				64	
	i.v. 100 cat units	10	64				128	
		30	64				128	
		45	128				256	

minutes after the injection of canine gallbladder bile (0.1 to 4 cc) into the main pancreatic duct. A further rise in amylase as well as in lipase concentration takes place later in peripheral and portal blood and lymph, the rise in peripheral and portal blood being about equal. Concentration of amylase in thoracic duct lymph is likewise increased. One-half to one hour after injection of bile it reaches usually higher values than in the blood. In Exp. 3 the portal vein was occluded above the inflow of the splenic vein for 10 minutes following the injection of bile into the pancreatic duct. In this experiment the amylase concentration in peripheral blood and lymph did not rise to any extent during the period of occlusion of the portal vein, while blood drawn from the latter showed considerable values for amylase and lipase. Amylase concentration in peripheral blood as well as in thoracic duct lymph rose considerably after release of the occlusion. In Exp. 4 and 5 intravenous injection of secretin was followed by an increase of amylase in lymph and blood whether the pancreatic duct was ligated or not. The concentration of amylase rose sooner and to higher

levels in the lymph than in the peripheral blood. Determinations of lipase were not done in all experiments, but whenever performed their relative changes in concentration followed closely those of blood amylase.

It is evident from our results that pancreatic enzymes reach the peripheral blood through the thoracic duct as well as through the portal vein. We believe that the main pathway is through the portal vein for the following reasons: The entire lymph from the thoracic duct is prevented from entering the peripheral blood in Exp. 1-2, and yet an increase of blood amylase occurs a few minutes following damage of the pancreas. This increase was observed also in one experiment in which the right main lymphatic duct as well as the thoracic duct were cannulated in the neck. Furthermore, the total amount of amylase reaching the circulation by way of the lymph is relatively small, because while the concentration of amylase in the lymph may be high, the factor of dilution in the blood stream has to be considered. This means that the greater part of the enzymes found in the blood in acute pancreatic disturbances passes through the portal vein and thus through the liver before reaching the general circulation. Further investigation shall deal with the question of whether the passage of enzymes through the liver may be the cause for such changes of the liver as are found frequently in acute pancreatic disease.

Summary. The concentrations of amylase and lipase in peripheral venous blood, portal blood, and thoracic duct lymph are equal in fasting anesthetized dogs. An increase of amylase in the blood often appears 5 to 10 minutes after damaging the pancreas. The increase of blood amylase and lipase following injury of the pancreas or retention of its secretion is due mainly to the inflow of pancreatic enzymes into the general circulation by way of the portal vein and to a smaller extent to inflow of enzymes by the lymph of the thoracic duct.

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An Agglutinable Factor in Human Blood Recognized by Immune Sera for Rhesus Blood.

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The capacity possessed by some rabbit immune sera produced with blood of Rhesus monkeys, of reacting with human bloods that contain the agglutinogen M has been reported previously.[1, 2] Subsequently it has been found that another individual property of human blood (which may be designated as Rh) can be detected by certain of these sera.

Upon exhaustion of such a serum with selected bloods, for instance OM, the absorbed serum still agglutinated the majority (39 out of 45) of other human bloods, independently of the group or the M,N type; moreover, reactions took place with bloods lacking the property P. An example of the reactions is given in Table I.

TABLE I.

	Bloods (all group 0)									
	Type M				Type N			Type M,N		
	1	2	3	4	5	6	7	8	9	10
Absorbed immune serum	+	+	+	0	0	+	+	+	0	+

Technic: Immune serum for Rhesus blood diluted 1:10, absorbed with half volume of sediment of blood 4. One drop each of absorbed serum, cell-suspension (2%) and saline used. Readings after 2 hours at room temperature. Positive agglutination designated by + sign.

The results are of some interest in that they suggest a way of finding individual properties in human blood, namely, with the aid of immune sera against the blood of animals. As an analogy may be cited the demonstration of differences in sheep erythrocytes with immune sera for human A blood.[3] The reactions described, although of moderate intensity only, were obtained with immune sera produced at different times. Whether these observations may possibly lead to a method suitable for routine work is still under investigation.

1 Landsteiner K., and Wiener, A. S., *J. Immunol.*, 1937, **33**, 19.

2 Wheeler, K. M., and Stuart, C. A., *J. Immunol.*, 1939, **37**, 169.

3 Andersen, J., *Z. f. Rassenphysiol.*, 1938, **10**, 104.